Asymmetric Reductions. XII. Stereoselective Ketone Reductions by Fermenting Yeast*

RODERICK MACLEOD,† HERBERT PROSSER, LOTTE FIKENTSCHER, JANOS LANYI, AND HARRY S. MOSHER

From the Department of Chemistry, Stanford University, Stanford, California

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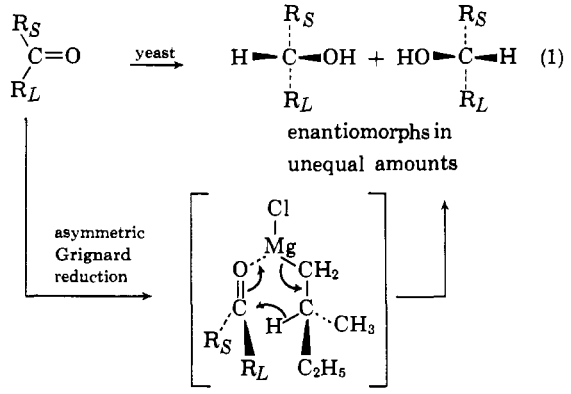
Values have been obtained for the stereoselective reduction by fermenting yeast of a series of ketones including all combinations of the substituents methyl, ethyl, *n*-propyl, *n*-butyl, and phenyl. Fermentation yields were improved by dispersing the insoluble ketone substrates on cellulose powder or Celite; isolation of highly purified optically active carbinols was made possible by use of gas chromatography. The enantiomorph of the carbinol produced in excess corresponded to the absolute s configuration, with one controversial exception, namely *n*-propyl-*n*-butylcarbinol. Stereoselectivity varied from values as high as 90% in the aromatic series to as low as 12% for ethyl *n*-propyl ketone. From this it is concluded that the difference in steric requirements of the two groups attached to the carbonyl function is the important factor in determining the configuration of the predominate transition state and not the absolute preference of one group or the other for a particular site on the enzyme surface. The lack of 100% stereoselectivity during fermentative reduction of these ketones can be rationalized by assuming that the reductions are catalyzed by alcohol dehydrogenase as the only enzyme involved but that it is only partially stereoselective when acting on these unnatural substrates.

The stereospecific asymmetric reductions of carbonyl substrates by enzyme-coenzyme systems such as the alcohol dehydrogenase–reduced diphosphopyridine nucleotide (ADH-DPNH)[1] system (Van Eys and Kaplan, 1957) and the stereoselective asymmetric reduction of carbonyl compounds by optically active Meerwein-Ponndorf (Streitwieser and Wolfe, 1959; Doering and Young, 1950) and Grignard reagents (MacLeod *et al.*, 1960) exhibit many interesting similarities. These have been the subject of recent reviews (Sund and Theorell, 1963; Westheimer, 1962; Kosower, 1962). Our knowledge concerning the precise details of these hydrogen-transfer processes in the chemical reductions is considerable; it would be valuable to know to what extent this information can be extended to the enzymic cases. Although both the chemical asymmetric reductions and the enzymic reductions



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give optically active products, those from the chemical systems have always been less than optically pure, while those from the enzyme process utilizing natural substrates have been optically pure as has been demonstrated so elegantly in the case of 1-deuteroacetaldehyde (Westheimer *et al.*, 1951). This difference is one of degree rather than of kind but it is a difference which we felt should be explored from both directions, namely, a search should be made for examples of the "chemical" asymmetric reduction process which might approach 100% stereoselectivity, and on the other hand a study should be made of enzyme processes, which when operating on something less than an ideal substrate, i.e., an unnatural substrate for which it has not been designed by nature, might exhibit something *less than* 100% stereoselectivity. Both approaches could give greater insight into the enzymic process.

Progress has been made on the first approach. The Grignard reagent from (+)-1-chloro-2-phenylbutane has caused an 82% asymmetric reduction of phenyl isopropyl ketone, i.e., 91% of the product was the *levo* and 9% the *dextro* isomer (Birtwistle *et al.*, 1964). Definitive results concerning the second point are lacking and it is this aspect of the problem with which we are primarily concerned in the present study.

Reductions of carbonyl compounds by purified ADH-DPNH systems indicate that a single enantiomorph is produced. But the only carbonyl reduction studied stereochemically with purified ADH-DPNH is that of 1-deuteroacetaldehyde. The fact that fermentative reductions proceed with absolute stereoselectivity with substances such as the steroids (Vischer and Wettstein, 1959) is well known and readily understood not only on the basis of the 100% stereoselective enzyme but also by virtue of the highly critical steric requirements of such asymmetric steroidal substrate molecules. Steric requirements are not as critical for compounds such as the isomeric decalones. Prelog and co-workers (Prelog *et al.*, 1959) have reported that the microbiological reduction of 2-decalone by *Curvularia falcata* is less stereoselective than that of 1-decalone and that both of the decalols produced were partially racemic. There is therefore reason to suppose that, if steric factors are determining, the simple ketones with lower steric requirements would result in a lesser degree of stereoselective reduction by such systems.

Numerous reductions have been carried out by the

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† U. S. Public Health Service Postdoctoral Fellow 1953–54; present address: Loyola University, Los Angeles, Calif.

[1] Abbreviation used in this work: ADH, alcohol dehydrogenase.

use of actively fermenting yeast (Neuberg, 1949) as an *in vivo* reduction system and in many cases *partially* active products have been obtained from the reduction of unsymmetrical ketones. For instance Neuberg and Nord (1919) reported that methyl ethyl ketone was converted by actively fermenting yeast to (+)-2-butanol (equation 1, R_S = methyl, R_L = ethyl) which possessed approximately 25% of the rotation of the pure *dextro* isomer.

This incomplete stereoselectivity by actively fermenting yeast is especially significant if the reaction proceeds substantially by way of a single enzymic reducing system. Since the steric course of the ketone reductions should depend upon the nature of the two substituents attached to the carbonyl group of the ketone, comparison of such effects for different ketones might then be expected to give direct information about the asymmetric environment of the transition state of the hydrogen-transfer process. Competing enzyme systems would, of course, interfere with a clear interpretation of the results. On the other hand, at least part of the reduction environment almost certainly is defined as the 4 position of the nicotinamide moiety of DPNH (or possibly TPNH) since no other dehydrogenase coenzymes are recognized. McKinley-McKee (1963) has proposed that the substrate-zinc-coenzyme conformation is dominant in determining the stereospecificity of the ADH-DPN system while the protein is not directly involved.

To obtain more reliable data, using refined methods of isolation and purification which were not available earlier when Neuberg and Nord did their pioneering experiments, we have undertaken a careful study of the stereoselectivity of actively fermenting yeasts toward unnatural ketone substrates. Ketones were chosen which would give the maximum amount of information, namely, a closed series in which all combinations of the substituents methyl, ethyl, *n*-propyl, *n*-butyl, and phenyl were studied.

In this study it was necessary to isolate rather small amounts of carbinols in a high state of purity from large amounts of fermentation mixtures. It was necessary to investigate the range of substrates which could be used as well as the stereoselectivity of the reaction and the conditions which would give the highest yields possible.

Experimental

Ketones.—These were commercially available and were subjected to rigorous fractionation and the purity was checked by infrared spectroscopy in each case. *Yeast:* Two types of fresh commercial baker's yeasts were used, Fleischmann's (Standard Brands, Inc.) and Red Star. The former was used in the earlier work and every ketone was reduced at least once using it. In the later studies on the alkyl aryl ketones the Red Star brand was used.

Procedure A.—These runs were completed before gas chromatography became available and thus the purity of the products was not known with the certainty possible with the technique developed in procedure B. The asymmetric reduction data are considered to constitute values of reasonable reliability and are certainly a great improvement over the earlier literature reports. The basic procedure is that of Neuberg and Nord (1919), as developed by Levene and Walti (1943) and adapted for the volatile products and, for the most part, water-insoluble substrates encountered in the present study. The major refinements of procedure A over that in the literature are the use of micro fractionation for purification and the use of infrared spectroscopy for establishment of identity and purity.

Fresh Fleischmann's yeast, 1800 g (4 lb), was dispersed to a smooth paste in distilled water (2 liters) at 35°. Glucose, 1800 g, was dissolved in 7.2 liters of hot water and allowed to cool to 30–33°. One-third each of the yeast slurry (1.2 liter) and of the glucose solution (2.8 liters) were combined (30–33°) in one of the three 10-liter glass fermentation bottles each of which was equipped with an efficient sealed stirrer, glycerine-filled gas trap, and provision for adding substrate and removing samples for analysis. The fermentations were maintained at a temperature of 34–36°. The ketone substrate, 10–20 g, dissolved in 10 ml of ethanol, was dispersed in 20 ml of a 2% aqueous suspension of Atlas surface-active agent G-672 and added to the fermentation mixture 20 minutes after it had become well started. After 48 hours a 600-ml slurry containing 300 g (2/3 lb) of yeast and 60 g of glucose, which had been allowed to stand for 20 minutes to initiate fermentation, was added to each. Twenty-four hours later, when there was a negative test for glucose, the mixture was processed as follows: The fermentation mixture was subjected to distillation at reduced pressure (bp 45–47°) until 2.5 liters of distillate had been collected. This crude distillate was then carefully fractionated through a helix-packed column (30 theoretical plates). The first fraction, bp 78–80°, was ethanol-water azeotrope (270–300 g); the second fraction, bp 80–100°, was collected at the end without reflux to allow steam distillation of water-immiscible products. This second fraction was saturated with sodium chloride and the oil layer was separated and dried with anhydrous sodium sulfate. Ether extraction with subsequent evaporation gave an oil which was primarily a mixture of substrate and its reduction product. The crude carbinol was then fractionated through a 60-cm semi-micro spinning-band column. Vacuum was used on all of the aromatic ketone distillations. The distillate boiling at the proper temperature for the carbinol was freed of residual ketone by treatment with 2,4-dinitrophenylhydrazine and the carbinol was recovered by steam distillation. A final microfractionation gave the purified carbinol. In each case the infrared spectrum of the purified carbinol was compared with that of an authentic *dl*- sample.

Procedure B.—The apparatus and general scheme for this procedure was the same as procedure A with the following variations. The fermentation vessel was a 12-liter Pyrex flask and the tube for introducing the substrate was modified to accommodate the addition of ketone adsorbed on powdered cellulose. The yeast employed was Red Star baker's yeast. Supplements of minerals were used in the proportions recommended by Olson and Johnson (1949) and thiamine (100 mg per run) in the amount found by Schultz *et al.* (1937) to be optimal for increasing the fermentation rate. If zinc ion was added it was incorporated in the form of zinc sulfate hydrate to the mineral supplement.

The ketone substrate, 5–15 g, was thoroughly dispersed on five times its weight of cellulose powder (or in a few cases, Celite). A prolonged period of vigorous shaking both during and after the dropwise addition of the ketone was necessary to ensure a uniform distribution of the ketone upon the adsorbent. The glucose (750 g dissolved in 3 liters of distilled water), yeast (670 g [1.5 lb] slurried in 750 ml of distilled water) and supplement (100 ml) were mixed and warmed to 35° in the fermentor. After the fermentation had proceeded for 20 minutes and was progressing

vigorously, the ketone in its adsorbed form was added. This caused a rapid release of supersaturated carbon dioxide. Carbon dioxide evolution was complete within 18 hours as a general rule. After the fermentation had stopped a second portion of glucose (750 g in 3 liters distilled water) and yeast (670 g [1.5 lb] in 750 ml distilled water) was added. Fermentation was at a slower rate because of the higher alcohol content (about 7%) of the fermentation mixture at this stage. After 1–4 days the mixture was brought to about 80% of saturation by the addition of salt (a saturated solution caused excessive bumping), and the mixture was steam distilled directly from the fermentor. The ethanol azeotrope recovered from the first liter of distillate in a typical run was 830 ml, corresponding to about 90% recovery of the theoretical yield of ethanol. Without salt saturation preceding the steam distillation only 50% of the ethanol was present in the first liter of steam distillate. Two additional liters of steam distillate were collected and redistilled separately as described previously. The crude carbinol from the evaporation of the combined ether extracts was purified by preparative gas chromatography (Beckman Instrument Co., Megachrom) using a Ucon Polar (Union Carbide Co.) on 60–80 mesh acid-washed fire brick packing in eight parallel columns 180 × 1.3 cm (72 × 0.5 in.) with helium carrier (at 180° for the alkylarylcarbinols). The gas-chromatographed final carbinol was checked for purity by analytical gas chromatography. If impurities were present the carbinol was rechromatographed on a single Ucon Polar column 152.4 × 0.95 cm (60 × 0.375 in.). The final carbinol was then distilled under high vacuum. This last distillation was essential since it removed traces of high-boiling Ucon Polar oil (and/or decomposition products from the oil) which caused a significant exaltation of the rotation of the carbinols.

Exaltation of Rotation of Carbinols Contaminated with Polyglycols from Gas Chromatographic Columns.—Highly purified (−)-*n*-butylphenylcarbinol which was 66% optically pure was mixed successively with 0.7, 1.3, and 2.6% optically inactive polyethylene glycol 300 (Carbowax). There was an *exaltation* in the rotations of 3, 5, and 8%, respectively, indicating strong component interaction. If only a dilution effect had prevailed a simple decrease in rotation would have been observed. Vacuum distillation successfully removed such high-molecular-weight column-"bleeding" contaminants from our samples. We were obliged to use this more lengthy purification procedure since the Ucon Polar columns were the only type available at the time which provided superior separations for our system components.

The strain of yeast was not controlled beyond the limits observed by the manufacturer and it is probable that some variability exists between batches of the same commercial yeast. It seems unlikely that this variation would be significant to these studies. A different yeast brand (Fleischmann) was used in procedure A than in procedure B (Red Star). Since there are several commonly employed baker's yeast strains it can be postulated that yeast strain differences account for the notably different results obtained in the reduction of acetophenone by the two procedures.

Results

Recoveries.—The total recovery of product carbinol plus substrate ketone from a fermentation mixture generally accounted for 60–70% of the substrate added when procedure A was used. In the case of the two most water-soluble and volatile ketones, 2-butanone and 2-pentanone, the recoveries were 30–60%. In two separate experiments with methyl *t*-butyl ketone neither starting material nor carbinol was isolated from the fermentations, and we hope to investigate this case further. Procedure B, a refined procedure used only on the more insoluble substrates, often gave an 80–100% recovery of crude products.

Yields.—The per cent reduction of ketones to secondary carbinols as listed in Table I was calculated in the aliphatic series on the basis of the weight and rotation of crude product and the determined rotation of the final purified carbinol. For the aromatic ketones reduced by procedure B the per cent reduction was determined from the gas-chromatographic peak area ratio of carbinol to total ketone plus carbinol in the crude oil isolated from the fermentation. The formation of each mole of secondary carbinol was accompanied by the formation of from 200 to 2000 moles of ethanol and therefore required 100–1000 moles of glucose per mole of ketone substrate reduced.

Methyl-*n*-butylcarbinol was the only carbinol isolated in high yield by procedure A. The use of procedure B consistently gave higher yields, especially for ethyl- and *n*-propylphenylcarbinols. This new procedure may therefore be of significant preparative value for obtaining various partially resolved carbinols. Such improved reduction procedures have recently been employed in these laboratories for the stereoselective reduction of deuterated aldehydes (Althouse and Mosher, 1961; Althouse *et al.*, 1960).

Although branched aldehydes are reduced by yeast (Althouse *et al.*, 1960; Senthe Shanmuganathan, 1960), branched aliphatic ketones are not generally reduced to an appreciable extent. As shown in Table I no successful reduction of phenyl *i*-butyl, phenyl *i*-propyl, phenyl *t*-butyl, or methyl *t*-butyl ketone was observed. It is also significant that the reduction of *n*-propyl *n*-butyl ketone was very inefficient.

Reproducibility of Results.—The data for the reduction of phenyl *n*-propyl ketone (Table I, five fermentations in which stereoselectivity varied by ±1%) amply indicate that the fermentations under controlled conditions are capable of giving a reproducible stereoselectivity. Nevertheless, it is apparent that some changes in fermentation conditions bring about significant differences in stereoselectivity. Because of the complexity of the *in vivo* system it is difficult to establish the critical variables responsible for the differences. The situation is further complicated by stringent requirements for sample purity since very small amounts of fusel oil components could severely affect carbinol rotations. Samples obtained by the refined purification and analytical methods of procedure B should be least subject to such variability. Trace amounts of fusel oil components can be completely separated from phenylcarbinols by gas chromatography and the observed rotation of samples obtained by procedure B are therefore believed to be highly reliable.

Effect of Added Zinc Ion.—Conflicting theories (Vallee, 1960; McKinley-McKee, 1963; Sund and Theorell, 1963; Barron and Levine, 1952; Van Eys *et al.*, 1957, 1958) exist concerning the role of zinc in the direct binding of substrate at the active site. The lack of zinc ion is reported (Miwa and Ueyama, 1960) to greatly inhibit sugar consumption by yeast. It was decided to determine if any effect of additional zinc in the fermentation medium could be observed as a systematic effect on the asymmetric reduction of ketones. The eight experiments on the reduction of phenyl *n*-butyl ketone which have from 0.02 to 1.0 g of added zinc ion (as $ZnSO_4 \cdot H_2O$) show no systematic

trend within the group. The only possibly significant result is an observed difference in asymmetry of 5% between two control runs (containing no added zinc) and the average asymmetry of the eight runs with added zinc.

It is possible that "saturation" of yeast with zinc can occur slightly above the levels of zinc found in commercially prepared baker's yeast. That such "saturation" would lead to a reduction of stereoselectivity cannot as yet be explained. Large differences in the zinc content of various baker's yeast strains have been shown to exist (Cook, 1958). The extensive work of Vallee (1960) and others on the zinc content and mechanism of action of zinc in yeast ADH has shown that extrinsic zinc does not exchange with intrinsic zinc in yeast ADH-purification procedures. Whether such exchange or "saturation" can occur in actively fermenting yeast is not known.

Substrate Solubility and Dispersion.—It would seem intuitively reasonable that the less water-soluble ketone substrates would be more poorly reduced by fermenting yeast. Poor solubility and cell permeability may be largely responsible for the small extent of reduction of *n*-amyl phenyl ketone. The better orientation of the methyl carbonyl function at the enzyme active site may provide a reason for the successful reduction of methyl nonyl ketone by Neuberg and Nord (1919). A method for providing a continually saturated solution of ketone throughout the fermenting mixture and for the duration of the fermentation is obviously highly desirable for the more-insoluble substrates. We have achieved this by the simple expedient of dispersing such substrates on inert adsorbents such as powdered cellulose. Greatly improved carbinol yields were obtained. The only reference we have found pertaining to the use of an inert high surface additive for improving the yield of a fermentation product involved the use of alumina in a bacterial fermentation (Bahadur and Ranganayaki, 1960). It has been stated (Thorne, 1957) that the presence of particles less than one-tenth the diameter of the yeast cell may restrict diffusion of metabolites thereby slowing growth and fermentation. Larger particles provide nuclei for the steady liberation of carbon dioxide and are thus considered to be beneficial to fermentation by helping to maintain yeast in suspension.

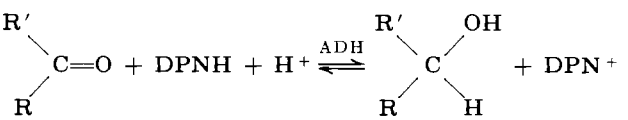
The data of experiments 33, 59, 60, and others show that the yield was not increased by starting with larger amounts of ketone in the fermentation. Optimum final yields of carbinol were obtained with 5–10 g of ketone per 8-liter fermentation, since isolation losses appeared to be minimized at this level. The final carbinols had approximately the same optical activity regardless of the amount of ketone employed.

Extent of Racemization During Fermentation and Isolation.—We can dismiss the possibility that racemization has occurred during isolation since this was checked by processing optically active ethylphenylcarbinol unchanged through the isolation procedure. We may also dismiss the possible presence of an enzyme which preferentially destroys one enantiomorph of the mixture of enantiomorphic secondary carbinols once formed since control experiments in which *dl*-methylphenylcarbinol and *dl*-ethylphenylcarbinol were subjected to the fermentation conditions gave back over 75% of the carbinols with rotations indicating less than 0.3% and 0.5% excess of the *dextro* isomers, respectively. The preferential oxidation of the *levo* carbinol would give this expected result. Since no appreciable amount of ketone was recovered and the optical activity of the recovered carbinols was very small it appears that the anaerobic fermentative reduction process is essentially irreversible.

Comparison with Previous Results.—Previously, Neuberg and Nord (1919) had reduced three of the ketones in Table I by actively fermenting baker's yeast. Whereas we obtained 64 and 67% asymmetric reduction in two experiments on methyl ethyl ketone, they reported a rotation which indicated 25% optical purity of their product. In the case of methyl *n*-propyl ketone we obtained 61 and 64% asymmetric reduction while they reported 46%; for methyl phenyl ketone we obtained 69, 70, 75, and 90% asymmetric reduction in five different experiments while they reported 36%. It is our belief, in view of the known difficulty of purifying the relatively small amounts of these liquids with the techniques available in 1919, that the earlier results represent the rotation of rather impure materials. We obtained in each case products with the same sign of rotation as those reported by Neuberg and Nord and there is therefore no conflict in the absolute stereochemical course reported.

Discussion

Substrate Specificity for the ADH-DPN System and for Actively Fermenting Yeast.—Although there has been considerable study of the relative reaction rates and substrate specificity for *oxidations* using the isolated yeast ADH-DPN (the reverse reaction of equation 2) (Van Eys and Kaplan, 1957; Levy *et al.*, 1957; Barron and Levene, 1952; Gierer, 1955; Müller, 1934) and liver ADH-DPN systems (Theorell and Bonnichsen, 1951; Nygaard and Theorell, 1955; Winer, 1958), there has been very little study of the substrate specificity for aldehyde and ketone *reductions* by the isolated ADH-DPNH system (the forward reaction equation 2) with the notable exception of the classical experiments on acetaldehyde and 1-deuteroacetaldehyde (Levy *et al.*, 1957; Westheimer *et al.*, 1951; Loewus



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et al., 1953; Fisher *et al.*, 1953) and the experiments reported by Senthe Shanmuganathan (1960) on the relative rates of reduction of various aldehydes. Kinetic data for the relative rates of *in vitro* enzymic reduction of ketones, even of acetone with yeast ADH-DPN, are not available.

The numerous substrates which have been reduced by actively fermenting yeast (Neuberg, 1949) stand in direct contrast to the limited number studied with the isolated ADH-DPNH system. The aldehydes which have been successfully reduced include compounds from the normal aliphatic, branched aliphatic, and aromatic series and include as examples *n*-valeraldehyde, α-methylbutyraldehyde, benzaldehyde, and phenylacetaldehyde. It is interesting to note that of these compounds *n*-valeraldehyde has also been reduced by cell-free yeast extracts (Neuberg, 1949, p. 79) and that the recent work of Senthe Shanmuganathan (1960) has shown that a series of normal and branched-chain aldehydes also can be so reduced.

Of the aliphatic methyl ketones the ethyl, *n*-propyl, *n*-hexyl, and *n*-nonyl homologs have been reduced by Neuberg and co-workers. Cyclopentanone, 2-methylcyclohexanone, and methyl phenyl ketone have also been reduced. Table I extends this series to include methyl *n*-butyl ketone, ethyl *n*-propyl and

TABLE I

KETONE REDUCTIONS BY ACTIVELY FERMENTING YEAST

$$R{-}\overset{\overset{O}{\|}}{C}{-}R' \longrightarrow RCHOHR'$$

Expt. No.	Ketone R	Ketone R′	Yeast Type and Procedure[a]	Ketone Weight (grams)	Carbinol Yield (%)[b]	Carbinol Refractive Index n^{20}_D	Carbinol Obs. Rotation, neat α^t_D (l = 0.5)	Carbinol Specific Rotation $[\alpha]_D$	Maximum Lit. Rotations Isomer[c] $[\alpha]^t_D$ max	Asymmetric Reduction[d] (%)	$\Delta\Delta G^{\ddagger}$ 30° (kcal/mole)[g]
1A	Me	Et	Fl-A	4.7		1.3872[e]	+7.53 (20)[e]	+17.3[e]	+25.43 (20)[e,f]	67	0.99
3B			Fl-A	6.0		1.3870[e]	+6.85 (20)[e]	+15.7[e]		64	0.92
3A	Me	*n*-Pr	Fl-A	7.2	20	1.4063	+3.49 (26)	+8.68	+13.70 (20)[g]	64	0.92
8B			Fl-A	7.2	18	1.4053	+3.34 (26)	+8.29		61	0.86
4A	Me	*n*-Bu	Fl-A	8.4	18	1.4138	+3.93 (20)	+9.60	+11.68 (20)[h]	82	1.74
1B	Me	*t*-Bu	Fl-A	7.8	0				+7.71 (20)[i]		
26			RS-B	10	0						
6A	Et	*n*-Pr	Fl-A	3.6	12	1.4124	+0.68 (26)	+1.67	+7.09 (20)[j]	23	0.28
7A			Fl-A	7.8	20	1.4128	+0.35 (27)	+0.86		12	0.14
4B	Et	*n*-Bu	Fl-A	9.5	8	1.4213	−0.73 (29)	−1.79	+6.7 (26)[k]	27	0.33
6B			Fl-A	9.5	4	1.4216	−0.36 (27)	−0.88		13	0.16
8A	*n*-Pr	*n*-Bu	Fl-A	5.4		1.4254	0.00	0.00	+0.57 (25)[k]	0	0
8B			Fl-A	5.4							
2A	Me	Ph	Fl-A	10	15	1.5232	−3.14[l]	−33.9[l]	+49.5[l,m]	69	1.03
2B			Fl-A	5	23	1.5230	−3.18	−34.3[l]		69	1.03
39			RS-B, 1 Zn[n,e]	5	45	1.5253	−19.72 (20)	−39.0	+43.5 (17)[m]	89	1.76
2B	Et	Ph	Fl-A	11.3	18	1.5232	−8.81 (26)	−17.88	−28.43 (26)[g]	63	0.89
5B			Fl-A	7.3	19	1.5230	−10.20 (27)	−20.73		72	1.12
29			RS-B, 1 Zn[n,p]	5	8	1.5175	−8.92 (25)	−18.02		64	0.91
30			RS-B, 1 Zn[n,p]	5	13	1.5181	−8.72 (26)	−17.62		62	0.88
6C	*n*-Pr	Ph	Fl-A	12.7	4	1.4882[e]	−46.41 (20)[e]		−102.6[e,q]	90	1.78
7B			Fl-A	11.5	5	1.4884[e]	−42.92 (20)[e]			84	1.50
34			RS-B[e,r]	5	40	1.4886[e]	−44.84 (20)[e]			87	1.65
33			RS-B,[e,r] 1 Zn	5	45	1.4888[e]	−44.39 (20)[e]			86	1.58
21			RS-B, 1 Zn	5	30	1.4890[e]	−44.12 (20)[e]			86	1.58
59			RS-B	10	19	1.4882	−12.11 (25)		−28.0[q]	86	1.58
60			RS-B	19	12	1.4885	−11.98 (25)			85	1.54
4C	*n*-Bu	Ph	Fl-A	13.6	5	1.5095	−7.17 (25)	−14.85	−17.17 (25)[r]	86	1.58
6C			Fl-A	6.1	7	1.5095	−7.42 (25)	−15.38		89	1.72
48			RS-B	5	37	1.5072	−5.50 (25)	−10.40		66	0.96
50			RS-B	5	26	1.5080	−5.48 (25)	−10.33		66	0.96
54			RS-B, 0.02 Zn[n]	5	19	1.5084	−4.80 (25)	−9.60		58	0.80
47			RS-B, 0.05 Zn	5	25	1.5076	−5.24 (25)	−10.95		63	0.91
49			RS-B, 0.05 Zn	5	35	1.5082	−4.98 (25)	−10.32		60	0.84
46			RS-B, 0.1 Zn	5	25	1.5080	−5.01 (25)	−10.38		60	0.84
51			RS-B, 0.1 Zn[s]	5	24	1.5080	−4.90 (25)	−10.15		59	0.82
52			RS-B, 0.2 Zn[s]	5	35	1.5080	−5.25 (25)	−10.89		63	0.91
53			RS-B, 0.5 Zn	5	20	1.5080	−4.94 (25)	−10.24		60	0.84
40			RS-B, 1.0 Zn[s]	5	21	1.5071	−5.06 (25)	−10.48		61	0.86
55			RS-B[t,s]	5	12	1.5080	−6.68 (25)	−13.83		81	1.25
56			RS-B[t,s]	5	9	1.5077	−6.24 (25)	−12.92		75	1.17
36	*i*-Bu	Ph	RS-B	7	0[u]				−32.3 (26)		
28	*n*-Am	Ph	RS-B	7	0[u]						
43	*i*-Pr	Ph	RS-B	7	0[u]						

[a] Yeast type F1 refers to commercial Fleishmann's yeast, RS refers to Red Star brand. Procedures A and B are described in the experimental sections. Deviations from the standard procedure are indicated with special footnotes. [b] These are estimated yields. For the aliphatic ketones these yields are based on the percentage carbinol present as determined by rotation of distilled fractions containing predominantly carbinol and ketone. For the aromatic ketones these yields represent the ratio of carbinol to carbinol-plus-ketone as determined by gas chromatography of the crude product. [c] The maximum value for the rotation of the neat liquid reported in the literature unless otherwise noted. The sign of rotation is that for the carbinol with the absolute s configuration as deduced from the data collected by Freudenberg (1933; p. 659) and discussed by Klyne (1954). [d] Per cent asymmetric reduction is obtained from $100 \times [\alpha]_{obs}/[\alpha]_{max}$ and represents the percentage excess of preponderant isomer. [e] Values for the acetate derivative instead of the carbinol. The acetates were prepared to facilitate isolation or if the carbinol was a crystalline solid. [f] Pickard and Kenyon (1914), p. 853. [g] Pickard and Kenyon (1911), p. 49. [h] Pickard and Kenyon (1911), p. 58. [i] Pickard and Kenyon (1914), p. 1120. [j] Kenyon and Poplett (1945). [k] Levene and Kuna (1941). [l] Rotation was not α_D, l = 0.5, neat, but $[\alpha]_D^{28}$ benzene, c = 7.11. [m] Downer and Kenyon (1911). [n] Additional zinc ion in grams added to the fermentation. The first four experiments on *n*-butyl phenyl ketone had no added zinc ion while the next eight had increasing amounts up to 1 g. [o] Additional thiamin, 100 mg, was added to these fermentations. [p] Dodecylamine, 3 g, used in addition to cellulose powder for dispersing the substrate. [q] Kenyon and Partridge (1936). The α_D for the acetate was obtained by extrapolation from values given for other wavelengths. The values for the undiluted partially active carbinols below their melting points were obtained on supercooled liquids and compared to extrapolated values from the literature. [r] Levene and Marker (1932). [s] The substrate was added in dilute alcohol solution as in procedure A, not adsorbed on cellulose. [t] Experiments 55 and 56 were without added zinc ion, but Celite replaced the cellulose as adsorbant for the added substrate. [u] A fair recovery of substrate ketone was achieved but it contained insufficient carbinol for isolation. [v] Calculated from $\Delta\Delta G^{\ddagger} = RT \ln k_S/k_R$.

ethyl *n*-butyl ketones, as well as the ethyl, *n*-propyl, and *n*-butyl homologs of the alkyl phenyl ketone series. We were unsuccessful in our attempts to extend these yeast reductions to include *n*-propyl *n*-butyl ketone, methyl *t*-butyl ketone, or isopropyl, isobutyl, *t*-butyl, or *n*-amyl phenyl ketones.

Configuration.—If the same steric forces which control the course of chemical asymmetric reductions (Eliel, 1962, pp. 68–78) also control the course of these yeast reductions, then one would predict that the preponderant isomer in each of the yeast reductions would have the same absolute configuration. The fact that eight of the nine secondary carbinols produced by reductive yeast fermentation of the corresponding ketones have the same configuration as represented by equation (1), where R_S represents the smaller group and R_L the larger group, indicates that steric factors are crucial in determining the stereochemical course of the yeast reductions of these ketones. Furthermore, the (+)-1-deuterobenzyl alcohol, (+)-1-dueterobutyl alcohol, and 1-deuteroneopentyl alcohol (equation 1, R_S is deuterium and R_L is phenyl, *n*-butyl and *t*-butyl, respectively), whose formation was previously observed in the yeast reductions of the corresponding aldehydes (Althouse and Mosher, 1961; Althouse *et al.*, 1960), belong to the same s-configuration series as required by the assumption of steric control of the course of this reaction. In addition it is s-(+)-2-octanol (equation 1, R_S is CH_3, R_L is *n*-C_6H_{13}) and not the enantiomorph which serves as a substrate for the purified ADH-DPN system (Van Eys and Kaplan, 1957); it is also the same isomer which is produced in preponderant amount by the reductive fermentation of 2-octanone (Neuberg and Nord, 1919, p. 2741).

The sole exception to the formation of a carbinol with s configuration in excess is the yeast reduction of ethyl *n*-butyl ketone from which was obtained (−)-ethyl-*n*-butylcarbinol whose relative configuration was established by Levene and Haller (1929) as being opposite to that of the (+)-methylalkylcarbinols produced in the other reductive fermentations of methyl alkyl ketones. Application of Freudenberg's displacement rule supports this assignment (Freudenberg, 1933). However Kenyon and Snellgrove (1925) obtained results opposite to those of Levene and Haller and thus there exists some room for doubt as to the finality of this configurational assignment, which can be settled only by further experimental evidence. Regardless of the correctness of the configurational assignment, the case of the asymmetric ethyl *n*-butyl ketone reduction brings two primary alkyl groups into steric competition and as might be expected the ability of the system to discriminate between two groups with the same amount of branching on the *alpha* carbon atom is very poor. As a result the stereoselectivity in the yeast reduction of ethyl *n*-propyl and ethyl *n*-butyl ketones was the lowest observed, corresponding to only about 0.2–0.3 kcal/mole difference in the energies of activation ($\Delta\Delta G^{\ddagger}$) between the competing *d*- and *l*-transition states.

The fact that the reduction of 1-deuteroacetaldehyde by purified yeast ADH–DPNH and the reduction of 1-deuterobenzaldehyde, 1-deuterobutyraldehyde, 1-deuterotrimethylacetaldehyde, methyl alkyl ketones, and phenyl alkyl ketones by actively fermenting yeast give carbinols in which the s configuration predominates is in accord with, but does not prove, the assumption that the same enzyme system is responsible for the *in vitro* as well as the *in vivo* reductions. The findings of Lemieux and Giguere (1951), that actively fermenting yeast gave β-hydroxy acids of opposite configuration

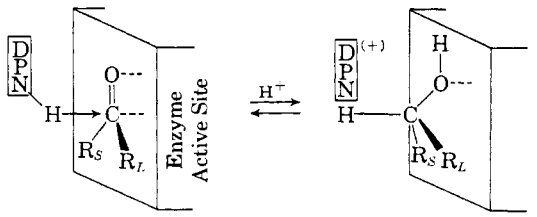


FIG. 1.—Stereospecific reduction of a carbonyl compound giving s enantiomorph. Oxygen function is chemically bound but R_S and R_L are located by "steric fit."

when β-ketocaprylic and β-ketocaproic acids were reduced, certainly render equivocal any conclusions based solely on such correlations.

The stereochemistry of the reduction of these carbonyl substrates can be represented schematically by Figure 1, where the hydrogen is transferred to the carbon of the carbonyl group from its "open" side. The binding site holds the polarized carbonyl group in a fixed orientation (Westheimer, 1962). In contrast, the orientation of the *substituents* on the ketone carbonyl group is determined by steric factors and not by primary binding forces. The steric fit of groups attached to the carbonyl carbon reaches an absolute selection for the case of the natural ADH substrate acetaldehyde and possibly for all other aldehydes. For *ketones* it is proposed that the receptive positions possess enough molecular freedom that they may accommodate either large or small groups (R_L and R_S). The extent to which the reaction goes faster with one orientation than the other determines the stereoselectivity of the reaction. It is important to note that this representation and the assumption that ADH-DPNH is involved in ketone reduction leads to the conclusion that it is the *difference in steric requirements* of R_S and R_L groups which is important and not any absolute preference of one group or the other for a particular enzymic site. Thus in the case of the natural substrate acetaldehyde R_S and R_L are hydrogen and methyl, respectively. According to this postulate the position occupied by hydrogen in acetaldehyde reduction is now the position preferred by the methyl group in methyl ethyl ketone reduction while the ethyl group must now occupy the position preferred by methyl in the acetaldehyde reduction. The energies of activation between the *d*- and *l*- transition states for ketones will not be separated by as high an energy barrier as for acetaldehyde and the over-all rate of reduction would be expected to be considerably slower. The slow rate of reduction of unnatural aldehyde substrates by ADH-DPNH has been shown by Senthe Shanmuganathan (1960).

When a bifunctional substrate, such as an α-keto acid, is reduced by an enzyme-coenzyme system, such as lactic dehydrogenase–DPNH, the substrate presents *two* primary binding sites to the system in a way not possible for a simple ketone with one functional group and inert alkyl substituents; thus the absolute stereospecificity can be determined regardless of the alkyl substituent in the case of the bifunctional substrate but not in the case of the monofunctional carbonyl compound.

Comparison of Stereoselective Reductions by Yeast and Optically Active Grignard Reagents.—A comparison of the trends in stereoselectivity between the actively fermenting yeast reductions and the reductions by optically active Grignard reagents (equation 1) as reported in Table II has been limited because of the inherent difficulties of both systems. On the one hand it has been found that the yeast will not reduce the more hindered branched-chain ketones which the Grignard reagent reduces most efficiently; and on the other hand the Grignard reagent will not give significant reduction of the unhindered aliphatic ketones which the enzyme handles best.

At the present time the substrates which have been reduced by both systems are listed in Table II. Because of the limited variety it is difficult to make any correlations beyond the fact that in each case the absolute configuration of the preponderant isomer is the same and is the one predicted for the chemical reduction based on its known mechanism (equation 1) and the relative steric requirements of the substituents. In every case the yeast system shows much higher stereoselectivity.

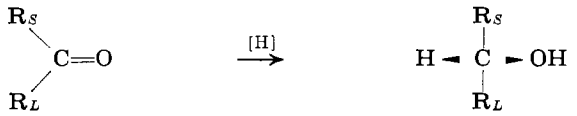
Stereoselectivity of Carbinol Oxidation by ADH-DPN.—In the reverse reaction, namely, the oxidation of various carbinols with yeast ADH-DPN, Van Eys and Kaplan (1957) have observed that approximately one-half the stoichiometric amount of DPN was consumed in the oxidation of *dl*-2-butanol and *dl*-2-octanol by purified ADH-DPN. In the case of 2-octanol the (+) isomer consumed the quantitative amount of DPN while the (−) isomer was not oxidized, thus demonstrating the complete or almost complete stereoselectivity of the oxidation.[2] However, Neuberg and Nord have subjected 2-octanone to actively fermenting yeast and obtained (+)-2-octanol of the predicted configuration but of only 35% optical purity. Furthermore, we have found that actively fermenting yeast *reduces* methyl ethyl ketone to s-(+)-2-butanol of 64–67% optical purity while the isolated ADH-DPN *oxidation* is specific for (+)-2-butanol as demonstrated by Van Eys and Kaplan (1957). Either a completely different system is involved in the purified yeast ADH-DPN secondary carbinol oxidations and the actively fermenting yeast ketone reductions, or the principle of microscopic reversibility does not hold here because of the differences of conditions involved. It has already been demonstrated by Van Eys and Kaplan (1957) that the geometry of reduced DPN is markedly different from that of the oxidized form and these authors have discussed the differences which may pertain when one or more of the coenzyme molecules associated with the enzyme are in the reduced form. The reduction of acetaldehyde usually has been conducted at a *p*H of about 7 while the oxidations of alcohols have been at a higher *p*H (8.0–9.5). The detailed conformation of the transition state and the concomitant stereoselectivity of the reaction might be considerably affected by the binding or lack of binding of a proton or protons to a critical point on either the enzyme or coenzyme by such a difference in *p*H.

Alternate Pathways of Carbonyl Reduction by Fermenting Yeast.—The postulate that the ADH-DPNH system is responsible for ketone reductions in actively fermenting yeast requires an explanation for the fact that actively fermenting yeast will reduce such substrates as trimethylacetaldehyde, methyl phenyl ketone, and *n*-butyl phenyl ketone while purified yeast ADH-DPNH will not reduce these at a measurable rate. It is conceivable that there is a different organization of the *in vivo* versus the *in vitro* enzyme system which results in a different stereoselectivity inside and outside the cell. No proof that it is the ADH-DPNH system which brings about these reductions exists and

[2] The precision of the results reported by Van Eys and Kaplan (1957), was probably such that the difference between 98 and 100% stereoselectivity might not have been detected.

TABLE II

STEREOSELECTIVE REDUCTIONS OF CARBONYL SUBSTRATES BY ACTIVELY FERMENTING YEAST AND OPTICALLY ACTIVE GRIGNARD REAGENT



Carbonyl Compound	Per Cent Asymmetric Reduction[a] Yeast[b] (%)	Grignard Reagent $(+)C_2H_5{*}CHCH_2MgCl$[c] (%)	Grignard Reagent $(+)C_2H_5{*}CHCH_2MgCl$[d] (%)
$(CH_3)_3CCDO$	100[e]	12[f]	
C_6H_5CDO	100[e]	22[g]	
$C_6H_5COCH_3$	90	3.9	37.4
$C_6H_5COC_2H_5$	61–63	5.7	52.2
$C_6H_5COC_3H_7$-*n*	85–87	5.9	
$C_6H_5COC_4H_9$-*n*	58–65	7.2	
$C_6H_5COC_4H_9$-*iso*	[h]	29.9	53
$C_6H_5COC_3H_7$-*iso*	[h]	24.0	82
$C_6H_5COC_4H_9$-*t*	[h]	16	15

[a] Per cent asymmetric reduction is the observed rotation divided by the maximum rotation times 100. [b] This paper, procedure B. [c] See MacLeod *et al.* (1960). [d] See Birtwistle *et al.* (1964). These results are in ether solvent at 25–35°. [e] These 1-deutero alcohols obtained by yeast reductions are assumed to be one enantiomorph but no independent evidence bears on this conclusion at present. [f] See Althouse *et al.* (1961). [g] See Althouse *et al.* (1960). [h] The branched-chain ketones failed to give sufficient carbinol for isolation.

one is forced to explore the possibility that another enzyme system is involved.

The alternative of other enzymes' being involved in carbonyl reduction by fermenting yeast is susceptible to experimental verification. The stereoselectivity of ketone reduction by cell-free yeast extracts as compared with actively fermenting yeast may prove enlightening. No such reduction of ketones has been reported as yet although, as previously mentioned, several aldehydes have been so reduced. Significantly, Fischer (1936) obtained a *levo* tiglic alcohol by reducing tiglic aldehyde (both carbonyl group and double bond are reduced) with actively fermenting yeast. He obtained a *racemic* mixture when a yeast extract was used. Although in this case the center of asymmetry was introduced during the reduction of the carbon-carbon double bond, here is an example of a difference in stereoselectivity between yeast *in vivo* and *in vitro* reductions.

Other known enzyme systems which might be operating in actively fermenting yeast cultures, and which might be competing with the ADH-DPNH system and thus account for partial stereoselectivity toward ketone substrates, include α-glycerophosphate dehydrogenase and α-hydroxyacid dehydrogenases. The *Saccharomyces cerevisiae* strains of brewer's and baker's yeast use the glycerol pathway for the fermentation of glucose to a small extent. Yields of glycerol up to a few per cent of the sugar consumed have been observed (Cook, 1958, p. 339). The effect of the glycerol pathway can be tested, since it is favored by an alkaline medium and by the presence of sulfite ion. Some strains of yeast, for example, *S. torulopsis* (Onishi, 1960), are also normally capable of converting glucose to glycerol in 30–40% yields. The glycerol pathway involves the reduction of the ketone group of dihydroxyacetone and the enzyme responsible for this step in the alternate glycerol pathway might be involved in the reduction of the unnatural ketone substrates. No reports on the substrate specificity of α-glycerophosphate dehydrogenase toward ketones nor the use of these high glycerol-producing yeast strains for reducing such substrates were found.

Yeast ADH can use D-(−)-lactic acid as a substrate although not very efficiently (Van Eys and Kaplan, 1957) (rate of oxidation of 3 versus 1000 for ethanol); however there is recent evidence (Nygaard, 1963) that there may be a lactic dehydrogenase system in yeast and that it is not stereospecific. The rate of oxidation of L-(+)-lactic acid was fifteen times that of the D-enantiomorph. The recent findings of Winer (1958) on the wide substrate acceptance of purified equine liver ADH makes this an interesting possibility for further investigation. A D-α-hydroxyacid dehydrogenase has also been described (Boeri *et al.*, 1960) as present in both anaerobic and aerobic yeast cultures, which reduced pyruvic acid when coupled with reduced flavin mononucleotide. Jakoby and Friedericks (1962) have isolated a secondary ADH from a species of *Pseudomonas* and the presence of a similar enzyme in yeast may have escaped detection.

In our experiments commercial baker's yeast was used and the possible effect of different yeast strains must be considered. It is reported that highly purified ADH from baker's yeast is different from that isolated from brewer's yeast (Keleti, 1958a) in turnover number, isoelectric point, amino acid content, and the kinetics of the DPN reduction. An ADH was isolated by Elisuzaki and Barron (1958b) from brewer's yeast which was much more active than the classical yeast ADH for ethanol and for other lower primary alcohols. Thus there is every reason to expect differences in the ADH of various strains of baker's yeast, although these differences should be small.

A final possibility, suggested by Keleti (1958b), is that yeast ADH contains three distinct kinds of active sites. He postulated that the "primary" sites are stereospecific for aldehydes while different sites are involved in the oxidation of *branched*-chain alcohols. These less-stereoselective sites could be further involved in the reduction of ketones. Thus one enzyme could retain complete stereoselectivity for aldehydes while demonstrating partial stereoselectivity toward ketones (with the exception of highly sterically oriented ketone systems such as the steroids). Keleti postulated that the secondary sites function without zinc atoms. These suggestions are still speculative at the present state of knowledge although the existence of more than one

type of active site in ADH is supported by some experimental data (Keleti, 1958b).

Conclusions.—We thus have at least three alternative explanations to consider for partial stereoselectivity of ketones by fermenting yeast. *First*, the yeast ADH-DPNH system is responsible for ketone reductions and, although it shows absolute stereoselectivity with its natural substrate acetaldehyde and possibly with other aldehydes such as 1-deuterobenzaldehyde and 1-deuterotrimethylacetaldehyde, it is only partially stereoselective with unnatural ketone substrates. *Second*, there is an as yet unrecognized carbonyl reductase in actively fermenting yeast which can utilize a wide spectrum of substrates and which has a lower order of stereoselectivity. *Third*, there exists more than one carbonyl reductase (possibly including ADH) in actively fermenting yeast which produce carbinols of opposite configuration but at different rates thus leading to the production of unequal amounts of the enantiomorphic carbinols.

We feel that the first of these alternatives is an attractive rationalization of the results to date. Until a large enough quantity of one or more unnatural substrates such as methyl ethyl ketone is reduced with an isolated ADH-DPNH system so that the carbinol may be isolated in a pure state and its rotation compared to that of the known optically pure enantiomorph, the first question cannot be answered definitively. At present, absolute stereoselectivity for reduction by the *in vitro* purified ADH-DPNH system has been demonstrated unequivocally only for 1-deuteroacetaldehyde.

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