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Stereochemical Aspects of the Substrate Specificity of Horse Liver Alcohol Dehydrogenase*

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ABSTRACT: The steric course of reduction of a number of 3- and 4-alkylcyclohexanones and the four 10-methyl-2-decalones has been determined. Marked rate differences between the enantiomers in both the *cis*- and *trans*-decalone series allowed resolution of the *dl* pairs. The reduction of (−)-3-methylcyclohexanone, *l*-10-methyl-*trans*-2-decalone, *d*-10-methyl-*cis*-2-decalone, and *l*-10-methyl-*cis*-2-decalone proceeded stereospecifically while the other substrates gave mixtures of axial and equatorial alcohols. From a correlation of observed rates and steric specificity, areas of a substrate that are consistent with high enzymatic activity, and inhibition areas, have been defined. Quantitative inhibition factors, allowing prediction of reaction rates and the stereochemistry of reduction, have been derived in a number of instances and a mapping of the steric relationship of enzyme, coenzyme, and substrate in the neighborhood of the active site has been proposed.

A number of investigators have demonstrated that crystalline horse liver alcohol dehydrogenase (liver ADH)[1] efficiently catalyzes the nicotinamide–adenine dinucleotide (NAD)-dependent ketone–alcohol interconversion of many derivatives of cyclohexanone and cyclohexanol (Winer, 1958; Merrit and Tomkins, 1959; Prelog, 1963, 1964). Studies (Prelog, 1963, 1964) of the oxidation and reduction rates of 2-methylcyclohexanones, 2-methylcyclohexanols, and certain 1-decalones and 1-decalols established a 2-alkyl inhibitory effect and led to proposals concerning the relative location of the coenzyme and substrate in the ternary complex; the presumed importance of the nonbonded interactions of the substrate with the carboxamide group of the coenzyme was stressed as an important factor in the determination of substrate specificity.

In the present paper, the hitherto unexplored reduction path of 3- and 4-alkylcyclohexanones and of the four 10-methyl-2-decalones is reported. From a correlation of observed rates and steric specificity it has been found possible to define areas of a substrate that are consistent with high enzymatic activity and inhibition areas that lead to rate decreases. In a number of instances, quantitative inhibition factors that allow the prediction of reaction rates have been derived. From these results and from a number of examples that ap-

* From the Worcester Foundation for Experimental Biology, Shrewsbury, Mass. *Received June 24, 1965.* Supported by a National Institutes of Health grant (AM-4044) and an American Cancer Society grant (T-185).

[1] Abbreviations used are: liver ADH, horse liver alcohol dehydrogenase; NAD, nicotinamide–adenine dinucleotide; $NADH_2$, reduced NAD; nmr, nuclear magnetic resonance; vpc, vapor phase chromatography; ORD, optical rotatory dispersion.

TABLE I: Gas Chromatographic Analysis of Cyclohexanones, 2-Decalones, and Reduction Products.[a]

Parent Substrate	Column[b]	Temp (°C)	Retention time (min)		
			Ketone	Ax Alc	Eq Alc
4-Methylcyclohexanone	A	75	5.6	9.0	13.6
3-Methylcyclohexanone	A	75	5.9	9.0	13.6
4-Isopropylcyclohexanone	B	105	5.7	4.0	5.0
4-*t*-Butylcyclohexanone	B	88	15.6	11.6	14.0
trans-2-Decalone	B	105	12.7	10.0	11.4
10-Methyl-*trans*-2-decalone	B	115	11.8	9.0	10.4

[a] The instrument was a Perkin-Elmer 811 gas chromatograph. [b] Column A was a 6-ft glass column with 30% glycerol on Chromosorb P. Column B was a 6-ft glass column with 3% XE-60 on Gas Chrom Z. The helium flow was 30 cc/min in each case.

pear in the literature, a preliminary mapping of the steric relationship of enzyme, coenzyme, and substrate in the neighborhood of the active site has emerged, and it has been found possible to offer a comprehensive explanation of the steric specificity of this enzyme.

Experimental Section

Materials and Methods

Horse liver alcohol dehydrogenase was purchased from C. F. Boehringer as a crystalline suspension in phosphate buffer containing 10% ethanol. The suspension was dialyzed against 0.05 M Tris buffer (pH 7.4) and after removal of insoluble material, the solution was placed on a column of Sephadex G-100. Elution with the same buffer and pooling of central fractions with peak enzyme activity gave a stock solution containing about 1.8 mg/ml of protein which was stable for a number of weeks at 0°. Enzyme prepared in this manner was used for all kinetic runs. β-$NADH_2$ was obtained from the Sigma Chemical Co. The (±)-2-methyl-, (+)-3-methyl-, (±)-3-methyl-, and 4-*t*-butylcyclohexanone were products of the Aldrich Chemical Co. Cyclohexanone and 4-methylcyclohexanone came from Eastman Organic Chemicals and 4-isopropylcyclohexanone from K and K Laboratories, Inc. The cyclohexanone derivatives were all redistilled and checked for homogeneity by vapor phase chromatography (vpc).

Alkylcyclohexanol Standards. The (±)-3-methyl-, 4-methyl-, 4-isopropyl-, and 4-*t*-butylcyclohexanones were each reduced with lithium aluminum hydride in ether solution, and the resulting mixtures of alcohol epimers were used as standards for vapor phase chromatography (vpc) analysis of the incubation products. The assignment of retention times to the axial or equatorial alcohol followed from the greater percentage of equatorial isomer in the reduction mixture and consequently greater peak area in the gas chromatograph. The percentage of each isomer in the chemical reduction was in accord with that reported by earlier workers (Noyce and Denney, 1950; Eliel and Ro, 1957). Retention times, as listed in Table I, demonstrate that in each case the equatorial alcohol was retained longer than the axial isomer.

dl-trans-2-Decalone. A sample of *dl-cis-trans*-2-decalol[2,3] in acetone solution was oxidized with chromic acid in 8 N sulfuric acid. Water was added and the product, after isolation by ether extraction, was purified by silica gel thin layer chromatography (ethyl acetate–benzene 15:85).

dl-cis-2-Decalone was prepared by chromic acid oxidation of *dl-cis-cis*-2-decalol.[2,3]

dl-trans-2-Decalols. Reduction of *dl-trans*-2-decalone with lithium aluminum hydride in ether gave a 77:23 mixture of equatorial alcohol (*dl-cis-trans*-2-decalol[2]) and axial alcohol (*dl-trans-trans*-2-decalol). The composition of the mixture was determined by thin layer chromatography and by vpc analysis.

dl-10-Methyl-trans-2-decalone was prepared by lithium–ammonia reduction of the corresponding α,β-unsaturated ketone as described by Yanagita *et al.* (1955).

dl-10-Methyl-cis-2-decalone. Reduction of the corresponding α,β-unsaturated ketone with palladium–carbon (Dauben *et al.*, 1954) gave the desired *cis*-decalone.

dl-10-Methyl-trans-2-decalols. A solution of 100 mg of *dl*-10-methyl-*trans*-2-decalone in anhydrous ether was reduced for 3 hr with 100 mg of lithium aluminum hydride. Saturated sodium sulfate solution was added dropwise until the precipitate began to adhere to the sides of the flask. Solid sodium sulfate was added, the solution was filtered, and the drying agent was thoroughly extracted with ether. The combined extracts were concentrated and the oily residue was chromatographed on silica gel thin layer plates (1 mm, ethyl acetate–benzene 15:85). Elution of the polar zone gave

[2] The first prefix defines the steric relationship of the C-2 hydrogen and the 9-hydrogen while the second prefix refers to the stereochemistry of the ring junction.

[3] This material was a generous gift from Professor Theodore Cohen, University of Pittsburgh.

80 mg of the equatorial alcohol (*dl-cis*-10-methyl-*trans*-2-decalol[4]), m.p. 67–68° and the less polar zone gave 11.2 mg of the axial isomer (*dl-trans*-10-methyl-*trans*-2-decalol[4]), m.p. 88–90° (Baker *et al.*, 1959).

dl-10-Methyl-cis-2-decalols. The chemical reduction of *dl*-10-methyl-*cis*-2-decalone was carried out as described for the 10-methyl-*trans*-2-decalone. The mixture of alcohols obtained in this manner was inseparable on thin layer chromatography and on vpc. In the nuclear magnetic resonance (nmr), the major methyl resonance peak of the mixture appeared at 56.5 cps and a minor methyl peak appeared at 57.5 cps.[5] On chemical grounds and on the basis of the methyl peak assignments of Musher (1961) the 56.5-cps absorption was assigned to *dl-trans*-10-methyl-*cis*-2-decalol[4] and the 57.5-cps resonance to *dl-cis*-10-methyl-*cis*-2-decalol.[4] The stereochemistry of the enzymatic reduction products was then determined by nmr in each case.

Resolution of dl-10-Methyl-trans-2-decalone. A solution of 23 mg of *dl*-10-methyl-*trans*-2-decalone contained in 2.0 ml of dioxane was dissolved in 300 ml of 0.03 M potassium phosphate buffer, pH 6.0. $NADH_2$ (100 mg) was added and reduction initiated by the addition of 50 mg of dialyzed (but not chromatographed) liver ADH contained in 10 ml of 0.05 M Tris buffer (pH 7.4). The reduction was carried out at 27° and followed spectrophotometrically by disappearance of the $NADH_2$ maximum at 340 mμ. Aliquots, taken at frequent intervals, were compared with a blank solution of identical concentration containing all components except substrate. Reduction, corrected for the blank, was allowed to proceed to about the 50% point (4 hr) when the rate of reaction was extremely slow. At this point the products were isolated by extraction with ether and subjected to thin layer silica chromatography (ethyl acetate–benzene 15:85) yielding (a) 6.8 mg of unreduced ketone and (b) 9 mg of *trans*-10-methyl-*trans*-2-decalol. The alcohol was reoxidized with chromic acid–acetone–8 N sulfuric acid and shown to be the *l* enantiomer by comparison of its optical rotatory dispersion with an authentic sample of *l*-10-methyl-*trans*-2-decalone.[6] The 6.8 mg of unreduced ketone (fraction a) was taken up in 0.6 ml of dioxane and, to ensure the complete removal of any remaining *l* compound, was subjected to a second reduction procedure (15.5 mg of enzyme, 31 mg of $NADH_2$, 100 ml of buffer). Reduction was allowed to proceed to about the 20% point (5 hr) when the products were isolated and separated by thin layer chromatography. The surviving ketone, 3.2 mg, was shown to be the *d* isomer by the positive Cotton effect in the optical rotatory dispersion curve; $[\alpha]_{307.5}^{CHCl_3}$ +1550° (Djerassi *et al.*, 1956). This material was used for the individual enantiomer rate studies. The degree of resolution is discussed under Results.

Resolution of dl-10-Methyl-cis-2-decalone. The reduction of 23 mg of ketone, contained in 2.0 ml of dioxane, was carried out as described for the *trans* isomer except that 25 mg of enzyme was used. After 6.5 hr, reduction had proceeded to the extent of about 40% when the reaction products were isolated by ether extraction and thin layer chromatography. Unreduced ketone, 9.0 mg (a), and *d-cis*-10-methyl-*cis*-2-decalol,[4] 8.1 mg (b), were obtained. The *cis* relationship of hydroxyl and methyl groups of (b) followed from the nmr which exhibited only a single methyl absorption peak at 57.5 cps. The alcohol was oxidized with chromic acid–acetone–8 N sulfuric acid to give, after purification by thin layer chromatography, 2.1 mg of *d*-10-methyl-*cis*-2-decalone: ORD $[\alpha]_{300}^{CH_3OH}$ −150°, $[\alpha]_{275}$ +358° (Djerassi and Marshall, 1958). The unreduced ketone (a) was subjected to reduction again with 25 mg of enzyme and 30 mg of $NADH_2$ in 110 ml of buffer. After about 20% reduction (3.5 hr), the mixture was extracted with ether and 2.5 mg of surviving *l*-ketone was obtained by thin layer chromatography: ORD $[\alpha]_{300}^{CH_3OH}$ +180°, $[\alpha]_{275}$ −335° (Djerassi and Marshall, 1958). The extent of resolution is discussed under Results.

Rate Studies. The reductions were followed by measuring the disappearance of $NADH_2$ at 340 mμ using a Cary Model 11 recording ultraviolet spectrophotometer. All reactions were carried out at 27° in 3-ml silica cuvets (1-cm light path) and in a total volume of 3 ml. The substrate in 0.02 ml of dioxane[7] and $NADH_2$ (final concentration 1.75×10^{-4} M) was added to 0.03 M phosphate buffer (pH 7.0) and the reaction was initiated by the addition of 0.1 ml of liver ADH solution containing 180 μg of protein. Suitable blank reactions containing all components but the substrate were run simultaneously, and the initial rate of reduction of each substrate was compared with that of cyclohexanone. Cyclohexanone and the alkylcyclohexanones were studied at a final concentration of 1.4×10^{-4} or 2.8×10^{-4} M. In the concentration ranges studied, and up to much higher concentrations, the rate of reduction of these substrates was found to be essentially proportional to the substrate concentration within the limits of the experimental errors in rate measurement.

For determination of the relative rate of reduction of the *cis*- and *trans*-decalones (with or without 10-methyl) substrate concentrations of 5.6×10^{-4} M were compared with cyclohexanone at the same concentration with the exception of *dl-cis*-2-decalone which was run at 1.4×10^{-4} M. The volume of dioxane was held constant at 0.02 ml in all runs. While recording periods

[4] The first prefix (*i.e.*, *cis* or *trans*) refers to the steric relationship of the angular methyl and hydroxyl substituents and the second prefix refers to the stereochemistry of the ring junction.

[5] Determined at 60 Mc/sec in CS_2 solution with tetramethylsilane as internal reference. The authors are grateful to Mr. John Cronan for nmr determinations.

[6] We are grateful to Professor Carl Djerassi, Stanford University, for a generous sample of the *l*-ketone which was used for the rate studies of individual enantiomers.

[7] In order to compare a variety of substrates, some of which were relatively insoluble, all substrates were dissolved in this volume of dioxane. With the methylcyclohexanone derivatives, the reduction rates relative to cyclohexanone and to each other were found to be essentially constant in the presence or in the absence of dioxane.

TABLE II: Relative Rate of Reduction of Alkylcyclohexanones.

Compound	Ref.[a]	Ref.[b]	This Study[c]
Cyclohexanone	100	100	100, 196[d]
(±)-2-Methylcyclohexanone	45	0.1	0.9, 1.95[d]
(±)-3-Methylcyclohexanone	36	50	50, 102[d]
(+)-3-Methylcyclohexanone		0.7	0.5
4-Methylcyclohexanone	80	40	41
4-Isopropylcyclohexanone			21
4-*t*-Butylcyclohexanone			2.5

[a] Merrit and Tomkins (1959); substrates 10^{-3} M. [b] Prelog (1963); substrates 10^{-2} M. [c] Substrates 1.4×10^{-4} M, $NADH_2$ 1.75×10^{-4} M, ADH 180 μg, pH 7.0, 0.03 M phosphate buffer, total volume 3.0 ml. [d] Substrates 2.8×10^{-4} M.

between 15 sec and 3 min sufficed for the faster substrates, it was necessary to extend the reduction period to 2 hr for the *l-cis-* and *d-trans*-decalones. The reduction rate for these two substrates was also determined by running the reaction with ten times the enzyme concentration (1.8 mg). For this purpose, chromatographed enzyme in phosphate buffer was prepared.

For comparison of the kinetic constants of cyclohexanone and benzaldehyde, runs were made as above but with only 1.8 μg of enzyme (contained in 0.01 ml). Separate runs were made with the substrates contained in 0.02 ml of dioxane or simply dissolved in phosphate buffer without dioxane. Cyclohexanone concentrations of 8.4×10^{-4} to 6.7×10^{-3} M and benzaldehyde concentrations of 1.02×10^{-4} to 6.2×10^{-4} M were utilized; K_m and V_{max} were determined by conventional Lineweaver–Burk plots with slopes and intercepts calculated by the method of least squares.

Product Formation. Reductions were carried out under the reaction conditions and with the substrate concentrations detailed in the rate studies. Depending upon the reduction rate, an aliquot was removed after a 10–30-min period and extracted with ether; the balance of the mixture was allowed to stand for a total period of 16 hr and then extracted. The (±)-3-methylcyclohexanone reduction was not allowed to stand since only the steric course of the (−) enantiomer was desired; similarly, the reaction of *dl-trans*-2-decalone was analyzed only in the early stages to ensure determination of products due only to the *d* enantiomer. Because of the very slow reduction rate of *d*-10-methyl-*trans*-2-decalone, the reaction was carried out with the usual substrate concentration of 5.6×10^{-4} M but with five times the usual enzyme concentration and ten times the usual $NADH_2$ concentration. The ether extracts were washed with water in each case, dried, and evaporated, and the residues were analyzed by vpc with the gas chromatographic systems listed in Table I.

Since in the case of the *cis*-decalones the product formation could be determined only by nmr, a larger scale reduction of *l*-10-methyl-*cis*-2-decalone was necessary. A solution of 2.3 mg of compound in 0.25 ml of dioxane was dissolved in 27 ml of phosphate buffer (pH 7.0). $NADH_2$ (25 mg) and dialyzed enzyme (25 mg) were added and the reaction mixture was incubated for 6 hr at 27°. After isolation by ether extraction and thin layer chromatography, 1.3 mg of reduction product was obtained. In the nmr, the material showed only a single methyl resonance peak at 56.5 cps, establishing the structure as *l-trans*-10-methyl-*cis*-2-decalol.[4]

Results

Relative Rate of Reduction of Alkylcyclohexanones. Table II compares the reduction rates of a number of 2-, 3-, and 4-alkyl-substituted derivatives of cyclohexanone. All substrates were compared at the relatively low concentration of 1.4×10^{-4} M and a relatively high $NADH_2$ concentration in order to demonstrate maximum differences between the various compounds. The general excellent agreement with the results of Prelog (1963) may be noted, while the major discrepancy with the data of Merrit and Tomkins (1959) lies in the rate of the (±)-2-methylcyclohexanone. A doubling of the concentration of three of the substrates also essentially doubled the initial rate of reduction, which demonstrates that the concentration range utilized was far below enzyme saturation. Both enantiomers of 2-methylcyclohexanone and (+)-3-methylcyclohexanone are seen to be poor substrates, while the relatively fast reduction of (±)-3-methylcyclohexanone must be attributed to the (−) enantiomer. A comparison of (±)-3-methyl concentration at 2.8×10^{-4} M and cyclohexanone at 1.4×10^{-4} M gave a relative rate of 102 for (−)-3-methylcyclohexanone. A progressive decrease in rate accompanied increasing size of a 4-alkyl substituent and became particularly marked with the 4-*t*-butyl group.

Relative Rate of Reduction of cis- and trans-2-Decalones. The resolution of the *d* and *l* enantiomers of the 10-methyl-*cis*- and -*trans*-2-decalones is described in the Experimental Section. While the ORD spectra and the vast rate differences between the resolved

enantiomers (shown in Table III) establish that the resolution is almost complete, the limited quantitative accuracy of ORD, in particular with the small amounts of substances that were available, does not allow calculation of the precise degree of resolution. Bearing in mind the proportionality of concentration and rate, an estimate may be made, however, on the basis of the relative reduction rates of the respective *d* and *l* isomers after resolution. It should be noted at this point that although a number of rate runs were carried out on each substrate, including runs at very high enzyme concentrations, the very slow rates of the *l-cis* and *d-trans* substances precluded a highly accurate relative rate determination and the rates reported in Table III

TABLE III: Relative Rate of Reduction of *cis*- and *trans*-Decalones.[a]

Substrate	Relative Rate
Cyclohexanone	100
dl-cis-2-Decalone	14.9
dl-10-Methyl-*cis*-2-decalone	2.2
d-10-Methyl-*cis*-2-decalone	4.0
l-10-Methyl-*cis*-2-decalone[b,c]	0.15
dl-trans-2-Decalone	2.2
dl-10-Methyl-*trans*-2-decalone	1.8
l-10-Methyl-*trans*-2-decalone	3.5
d-10-Methyl-*trans*-2-decalone[b,d]	0.035

[a] Reduction rates were compared with cyclohexanone at substrate concentrations of 5.6×10^{-4} M, $NADH_2$ 1.75×10^{-4} M, ADH 180 μg, pH 7.0, 0.03 M phosphate buffer, total volume 3.0 ml. The *dl-cis*-2-decalone was also compared at 1.4×10^{-4} M with cyclohexanone at 1.4×10^{-4} M. [b] Also run with 1.8 mg of ADH. [c] It is estimated that this compound contained 1.9% of the *d* enantiomer. The calculated rate for 100% resolved material is 0.075. [d] It is estimated that this compound contains 0.5% of the *l* enantiomer. The calculated rate for 100% resolved material is 0.0175.

are our best estimates, and may be off by as much as 25%. Table III shows that the relative reduction rate of the *l-cis* compound (0.15) was only 3.8% of the *d-cis* substance (4.0) and the rate of the *d-trans* (0.035) was only 1% of the *l-trans* compound (3.5). The resolution by reduction of two substrates (such as the *dl* enantiomers) which differ markedly in rate should continue until a steady state is reached where the concentration of the faster substrate times its relative rate is equal to the concentration of the slower substrate times its relative rate. Thus in each case, half of the observed reduction rate of the resolved slower enantiomer is due to the presence of a small amount of the faster enantiomer and, on this basis, it is easily demonstrated that the *l-cis*-decalone contains 1.9% of the *d-cis* compound and the *d-trans*-decalone contains 0.5% of the *l-trans*-decalone. While these considerations do not affect the rates of the faster enantiomers, the relative rate for 100% resolved *l*-10-methyl-*cis*-2-decalone should be 0.075 and that for 100% resolved *d*-10-methyl-*trans*-2-decalone should be 0.0175. In the discussion that follows and in the calculation of inhibition factors these latter figures are taken as the best estimate of the true rates of these two compounds.

It can be seen by comparing the reduction rate of *dl-cis*-2-decalone with *dl*-10-methyl-*cis*-2-decalone that the 10-methyl group had a pronounced inhibitory effect that was essentially absent in the *trans*-decalones (*dl-trans*-2-decalone *vs. dl*-10-methyl-*trans*-2-decalone.

Initial Product Formation. The initial products of enzymatic reduction as determined by gas chromatography and, in the case of the *cis*-decalones, by nmr, are listed for each substrate in Table IV. The figures in

TABLE IV: Initial Product Formation: Alkylcyclohexanones and -decalones.

Substrate	Configuration of Alcohol Formed[a] Equatorial	Axial
(+)-3-Methylcyclohexanone	91[b]	9[b]
(−)-3-Methylcyclohexanone	...	100
4-Methylcyclohexanone	70	30
	(78)[c]	(22)[c]
4-Isopropylcyclohexanone	65	35
	(82)[c]	(18)[c]
4-*t*-Butylcyclohexanone	5	95
	(45)[c]	(55)[c]
dl-trans-2-Decalone[d]	...	100
d-10-Methyl-*trans*-2-decalone	65	35
	(68)[c]	(32)[c]
l-10-Methyl-*trans*-2-decalone	...	100[b]
d-10-Methyl-*cis*-2-decalone	...	100[e]
l-10-Methyl-*cis*-2-decalone	100	...

[a] ... means formation was not observed. [b] No change in isomer ratio after overnight equilibration. [c] Product distribution after overnight reduction and equilibration. [d] Reflects reduction of only the *l* enantiomer. [e] The *cis*-decalols with the 10-methyl and 2-hydroxyl groups *cis* to each other are referred to as axial although the compounds with axial hydroxyl group are conformationally unstable.

parentheses denote product distribution determined after overnight reduction and equilibration. Of the alkylcyclohexanones, only (−)-3-methylcyclohexanone gave completely stereospecific reduction, while in the case of the 2-decalones only the reduction of the *d*-10-methyl-*trans* compound was not completely stereo-

specific. In contrast to 4-methyl- and 4-isopropylcyclohexanone, axial alcohol formation was heavily favored as the initial reduction product of *t*-butylcyclohexanone. The increase in the percentage of equatorial isomer which accompanied enzymatic equilibration when both isomers were initially formed was to be anticipated on the basis of the greater thermodynamic stability of the equatorial alcohols. This effect was particularly marked in the case of the *t*-butyl compound.

Kinetics of the Reduction of Cyclohexanone and Benzaldehyde. Table V compares the K_m and V_{max} values for the reduction of cyclohexanone and benzaldehyde when the substrates were dissolved in 0.02 ml of dioxane and in the absence of dioxane. Points for a Lineweaver–Burk double reciprocal plot were fitted by the method of least squares. In contrast to the benzaldehyde curves, the slopes of the cyclohexanone curves were extremely steep, as is evident from the high K_m values, and, therefore the intercepts (V_{max}) in the presence and absence of dioxane are not considered to be significantly different from each other or from the benzaldehyde intercepts. The V_{max} values for benzaldehyde are viewed with confidence and establish that dioxane, in the concentration used, is a competitive substrate inhibitor.

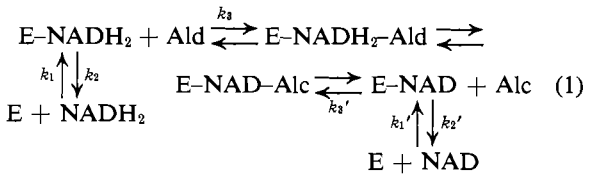
TABLE V: Kinetics of the Reduction of Cyclohexanone and Benzaldehyde with Liver ADH.[a]

Compound	V_{max} (ΔOD/min)	K_m (M)
Cyclohexanone	0.228	1.7×10^{-2}
Cyclohexanone in dioxane (0.02 ml)	0.345	5.1×10^{-2}
Benzaldehyde	0.267	9.7×10^{-5}
Benzaldehyde in dioxane (0.02 ml)	0.266	2.2×10^{-4}

[a] Reduction conditions: $NADH_2$ 1.75×10^{-4} M, ADH 1.8 μg, phosphate buffer, pH 7.0, 0.03 M, total volume 3.0 ml.

Discussion

Kinetic Significance of Differences in Rate of Reduction of Cyclohexanone Derivatives. The differences in the rate of reduction of the various cyclohexanone and bicyclic derivatives may be related to their rate of addition to the binary enzyme–$NADH_2$ complex and as such are a measure of steric "fit." This conclusion stems from the following considerations. The extensive kinetic studies with liver ADH, primarily with the acetaldehyde–ethanol system (*cf.* Theorell and McKinley-McKee, 1961) demonstrate that there is a preferential ordered reaction mechanism (1) whereby the coenzyme is bound to the enzyme before the alcohol or aldehyde substrate. While ternary complexes of enzyme–coenzyme–substrate are formed, interconversion of these



(1)

complexes (*i.e.*, the hydride-transfer step) is rapid and the over-all rate-limiting step at high substrate and coenzyme concentration is the dissociation of the coenzyme (k_2 or k_2') from the binary complex. The initial rate equation for aldehyde reduction derived by a steady-state treatment is given in eq 2 (Theorell and McKinley-McKee, 1961). Utilizing the values of the

$$\frac{e}{V_0} = \frac{1}{k_2'} + \frac{1}{k_1[NADH_2]} + \frac{1}{k_3[Ald]} + \frac{k_2}{k_1k_3[NADH_2][Ald]} \quad (2)$$

rate constants reported by Theorell and McKinley-McKee (1961) the expression at low substrate concentrations and $NADH_2$ concentrations of about 10^{-4} M reduces to eq 3 where the initial rate of reduction now becomes a function of the rate of dissociation of NAD (k_2') and the rate of formation of the ternary complex (k_3). With a very good substrate such as acetaldehyde, k_2' and k_3[Ald] are approximately of the same order of magnitude at an aldehyde concentration of *ca.* 10^{-4} M, whereas with poorer substrates such as cyclohexanone and its derivatives, the $1/k_3$[Ald] term becomes large relative to $1/k_2'$ and the initial rate at a fixed enzyme concentration depends only upon substrate concentration and the rate of ternary complex formation as described by eq 4. It may be noted that under these

$$\frac{e}{V_0} = \frac{1}{k_2'} + \frac{1}{k_3[Ald]} \quad (3)$$

$$\frac{e}{V_0} = \frac{1}{k_3[ketone]} \quad (4)$$

conditions a doubling of substrate concentration results in a virtual doubling of rate, as has been observed for cyclohexanone and the (±)-2- and -3-methyl derivatives. The tacit assumption that the over-all kinetic mechanism for liver ADH is the same with a ketone as with an aldehyde and that, in fact, the same active site is utilized by the enzyme for both substrate types has never been questioned, but this conclusion is supported by the following evidence. At a fixed concentration of enzyme and relatively high concentration of coenzyme the maximal velocity for the reduction of cyclo-

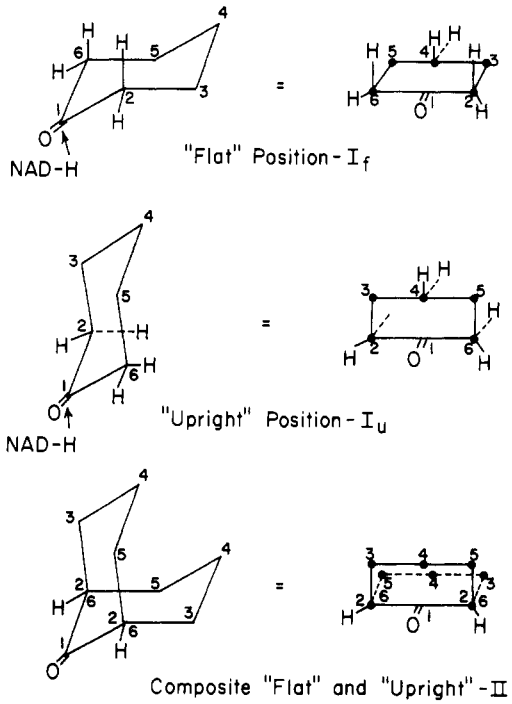


FIGURE 1: Representation of the two basic positions for reduction of a cyclohexanone derivative.

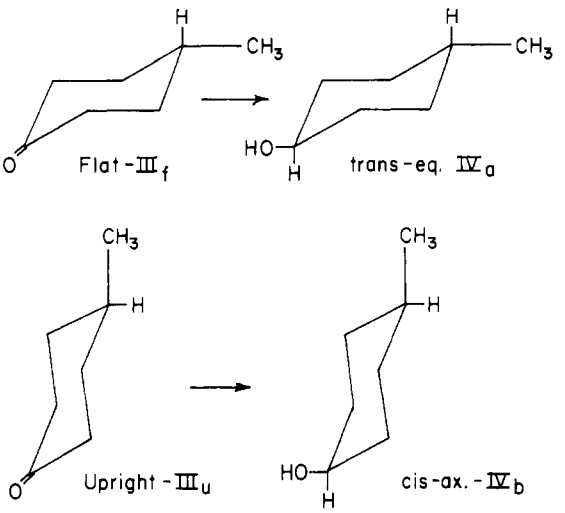


FIGURE 2: Demonstration of the dependency of equatorial or axial alcohol formation on the basic position of 4-methylcyclohexanone.

hexanone and for the excellent substrate benzaldehyde was the same within the range of experimental error. As noted in Results, this error stems from the extremely steep slope of the cyclohexanone curves. This indicates that for both substrates there is a common rate-limiting step at substrate saturation, specifically the dissociation of NAD from a common binary complex. Although these data do not prove the case, they are consistent with a common active site and kinetic reaction mechanism for both aldehydes and ketones.

On this basis the rate of reduction of the cyclohexanone derivatives has been related to their rate of binding to the enzyme–$NADH_2$ binary complex. From a combination of rate data and a knowledge of the stereochemistry of reduction a reasonably comprehensive picture has been formulated which illustrates certain steric interactions of enzyme, substrate, and coenzyme at the active site and the nature of enzyme "specificity" with liver ADH.

"Fit" of a Cyclohexanone Derivative. Assume a fixed position for $NADH_2$ in the enzyme–$NADH_2$–ketone ternary complex and, for optimal hydride transfer, a precise positioning of the carbonyl compound undergoing reduction *above* the dihydronicotinamide C-4 hydrogen atom. Then, in the ternary complex, two positions are possible for cyclohexanone and its derivatives which allow identical positioning of the carbonyl group and its two flanking carbon atoms (Figure 1). For convenience we designate the first of these as the flat position (If) shown schematically in side and front view. The upright position (Iu) is attained by standing the molecule upright and rotating it 180°. The composite diagram (II) illustrates the three carbon atoms occupying the same spatial position, but it should be noted that there is a juxtaposition of the C-2 and C-6 atoms in the two positions so that while C-2 and C-3 appear on the right in the flat position they are on the left in the upright position. Although this is unimportant in the case of cyclohexanone itself, a substituted cyclohexanone (*e.g.*, 2-methyl) will have the methyl group on opposite sides depending on whether the molecule is in the flat or in the upright position. Reduction of a cyclohexanone derivative in the flat position (If) will lead initially to the equatorial alcohol, while the upright position (Iu) will lead to the axial alcohol. Since cyclohexanol is conformationally mobile, the extent of axial alcohol formation will be concealed by immediate flip and equilibration to a mixture consisting predominantly of the equatorial alcohol; therefore the position in which cyclohexanone itself undergoes reduction cannot be determined. However, when the cyclohexanone bears an alkyl substituent, as for example 4-methylcyclohexanone (III) (Figure 2), both the starting ketone and the products of reduction are conformationally much more stable since the relatively large methyl group will prefer to remain in an equatorial position. More important, once reduction has occurred the relative position of the hydroxyl and methyl groups are fixed and the substance with methyl and hydroxyl on opposite sides (*trans*) is not convertible by ring flip to the product with the two groups on the same side (*cis*). Thus, determination of the ratio of *trans*(equatorial)- (IVa) to *cis*(axial)-4-methylcyclohexanol (IVb) defines whether reduction occurred in the flat (IIIf) or upright (IIIu) position. On this basis, the individual substituents may be examined in detail. It may be noted that although the reverse reaction, alcohol oxidation, was not extensively studied, a favorable or unfavorable steric position for ketone reduction

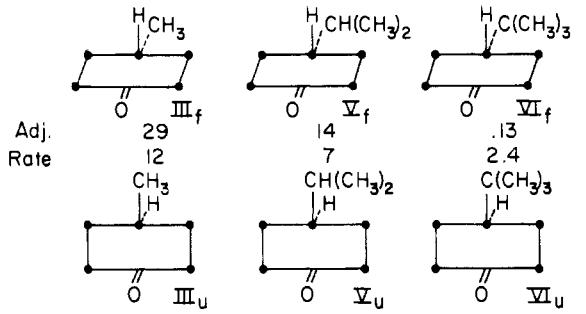


FIGURE 3: Reduction positions and adjusted rates for the 4-alkylcyclohexanones.

dictates a similar situation for alcohol oxidation since the oxidation–reduction is freely reversible and must pass through a common transition state.

The stereochemistry of reduction of the various substrates is detailed in the following sections. The data have been analyzed by treating each alkylcyclohexanone as fixed in its thermodynamically most favored conformation with the alkyl group occupying an equatorial position. Although the low steady-state concentration of axial conformers may make a small contribution to the over-all observed rate, there is no indication that a substrate may be "fixed" by this enzyme in an unfavorable high-energy conformation.

The adjusted rate which is assigned to the flat and upright position for each substrate is derived by multiplying the rate of reduction of the substrate relative to cyclohexanone (Tables II and III) times the fraction reduced in that particular position, as determined by the products of initial reaction (Table IV). It is arbitrarily assumed that cyclohexanone with a rate of 100 reduces equally in the flat and upright positions, so that an adjusted rate of 50 may be taken as standard for either position.

4-Alkylcyclohexanones. The favored positions (IIIf, Vf, and VIu) (Figure 3) from which 4-methyl-, 4-isopropyl-, and 4-*t*-butylcyclohexanone initially undergo reduction (flat, flat, upright) follow from the preponderance of *trans*(equatorial) alcohol formation with the first two substrates and *cis*(axial) alcohol with the *t*-butyl derivative. While these are favored positions the alternate positions are by no means forbidden, as shown by the substantial percentage of axial alcohol formation with the first two substrates. Even in the case of *t*-butyl with near steric specificity at an initial axial-equatorial ratio of 19:1, overnight equilibration led to a significant increase in the percentage of equatorial isomer (Table IV). *Therefore it is clear that reduction of a cyclohexanone derivative can occur in either the flat or upright position and both axial and equatorial alcohols are formed at significant rates.*

The rate decrease in going from 4-methyl to 4-isopropyl is only twofold, which indicates that the two additional side-chain methyl groups are not a serious steric bar. However, the additional eightfold rate decrease which accompanies the conversion of isopropyl into *t*-butyl points to a severe steric interference with ternary complex formation due to the one additional methyl group. If the reasonable assumption is made that the isopropyl and *t*-butyl groups lie in their thermodynamically most favored position with the side-chain methyl groups staggered relative to the ring methylenes (V and VI) (Figure 4), then the methyl group which leads to a rate reduction may be identified as R which projects below the molecule in the flat position (f) and in to the front of the molecule in the upright position (u). It is apparent from the adjusted rates of the isopropyl and *t*-butyl derivatives that the methyl group R decreases the rate of reduction by a factor of 108-fold in the flat position but only 2.9-fold in the upright position. The great favoring of axial over equatorial alcohol formation follows from the relative severity of this methyl interaction in the two positions.

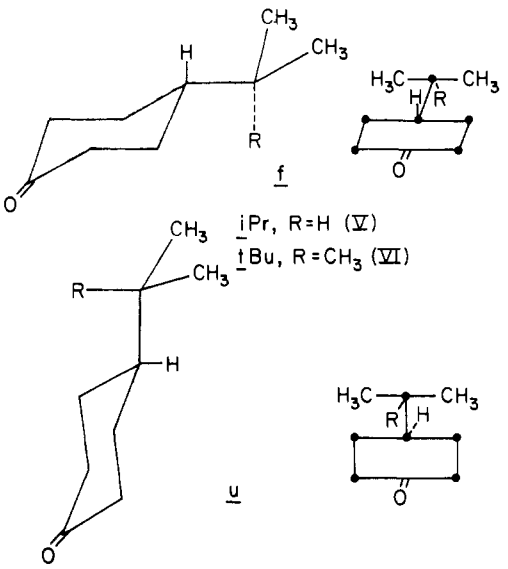


FIGURE 4: Conformational and schematic representation of 4-isopropyl- and 4-*t*-butylcyclohexanone.

While the diminished reduction rate of 4-methylcyclohexanone relative to cyclohexanone must stem from a steric interaction of the 4-methyl group, it cannot be demonstrated with certainty whether this is the result of interactions in both the flat and upright positions since the steric course of the reduction of cyclohexanone itself cannot be ascertained. It will be shown in the case of the *cis*-bicyclic derivatives, however, that a 4-methyl group in the upright position reduces the reaction rate by a factor of approximately seven. Based on a cyclohexanone rate of 50 in each position, the 4-methyl group slows the reduction rate of IIIf and IIIu by factors of 1.7 and 4.2, respectively.

3-Alkylcyclohexanones. Of the two possible (−)-3-methylcyclohexanone [(−) VII] positions (Figure 5), the fast reduction rate and the exclusive formation of the *trans*(axial) alcohol establishes the upright position u as highly favored and the flat position f as virtually

forbidden. Since the reaction rate of the (+)-3-methyl enantiomer [(+) VII] is so much slower than the (−) compound, reduction of (±)-3-methylcyclohexanone should lead to resolution by selective reduction of the (−) compound. Equatorial alcohol formation heavily predominates with the (+) compound; therefore the 3-methyl substituent in (+) VIIu is in a worse position with respect to substrate binding than in (+) VIIf. It is apparent though that an equatorial 3-methylcyclohexanone, or derivative thereof, must be positioned and substituted as in (−) VIIu to serve as a satisfactory substrate for liver ADH. Additional support for this conclusion comes from the finding (Prelog, 1964) that the alcohol derived from (−) VIIu undergoes oxidation with liver ADH at a rate equivalent to cyclohexanol while the alcohol derived from (+) VIIf reacts at less than 1% of that rate. These oxidative examples constitute part of Prelog's (1963, 1964) argument that an equatorial hydrogen is transferred to and from the substrate more readily than an axial hydrogen and therefore liver ADH should be labeled as an equatorial enzyme. As already pointed out in the 4-methylcyclohexanone discussion either equatorial or axial hydrogen transfer can readily occur; therefore the oxidation rate of a 3-methylcyclohexanol must be rationalized on the basis of a favorable or unfavorable interaction at the 3-methyl position rather than on the basis of axial or equatorial hydrogen transfer. Inhibition factors of 110, 1100, and >100 may be assigned to the 3-methyl group as positioned in (+) VIIf, (+) VIIu, and (−) VIIf. In contrast, the methyl group of (−) VIIu is not only consistent with high substrate activity, but actually increases the rate by a factor of two based on a cyclohexanone value of 50 in the upright position. It is emphasized again that the standard cyclohexanone adjusted rates of 50 in each position are assumed values. If, in fact, the reduction of cyclohexanone is favored in the upright position, the inhibition effect of the 4-methyl group in IIIf will be somewhat less than 1.7 and the rate stimulation of the 3-methyl group in (−) VIIu will be less than a factor of two. Inhibition factors calculated for the bicyclic derivatives are independent of cyclohexanone rate assumptions since the alkylcyclohexanones with known adjusted rates serve as standards.

10-Methyl-trans-2-decalones. The four possible positions of the enantiomeric (*d* and *l*) 10-methyl-*trans*-2-decalones, as represented in Figure 6 by *d*-VIIIf, *d*-VIIIu, *l*-VIIIf, and *l*-VIIIu, are each seen to bear a methylene group corresponding to a position found in the 3-methylcyclohexanones. The resolution of the racemic *trans*-decalones by selective reduction of the *l* compound and the stereospecific formation of the axial alcohol (angular methyl and hydroxyl groups *cis*) could have been predicted *a priori* on the basis that only *l*-VIIIu bears a 3-methylene group in the acceptable upright position (C-8, *l*-VIIIu, Figure 6) corresponding to the 3-methylcyclohexanone position (−) VIIu in Figure 5. Similarly, the preferential reduction (albeit slow) of the *d* isomer in position *d*-VIIIf rather than in *d*-VIIIu is in accord with the general pattern observed with (+)-3-methylcyclohexanone, although the position

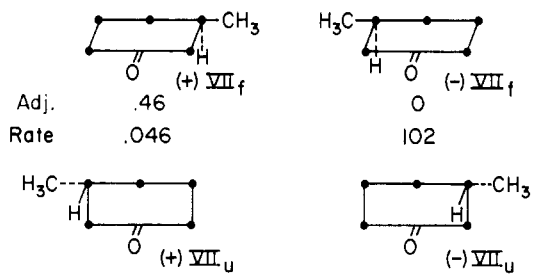


FIGURE 5: Reduction positions and adjusted rates for (+)- and (−)-3-methylcyclohexanone.

preference and consequent formation of equatorial alcohol is more marked in the monocyclic compound. Considering only the favored positions *d*-VIIIf and *l*-VIIIu for the *d* and *l* isomers it is seen that their reduction rates are reduced by 20- to 30-fold factors compared to the corresponding 3-methylcyclohexanones (+) VIIf and (−) VIIu. The steric factors which contribute to these rate differences will be analyzed following discussion of the *cis*-bicyclic compounds, but it may be noted at this point that the reduction rate of *dl*-*trans*-2-decalone was only 20% faster than *dl*-10-methyl-*trans*-2-decalone, which eliminates the angular 10-methyl group as a serious source of steric hindrance.

10-Methyl-cis-2-decalones. As in the case of the *trans*-decalones the *d*- and *l*-10-methyl-*cis*-decalones (Figure 7)[8] may be analyzed on the basis of a 3-methylcyclohexanone effect. The four possible positions are pictured with *d*-IXu being shown also in side view. With these *cis* derivatives, if the ring bearing the ketone function is in the upright position (*e.g.*, IXu), the second ring is in a flat position and *vice versa* (IXf). Products of reduction cannot be simply described as axial or equatorial alcohols, however, since the "axial" *cis*-decalols are conformationally unstable (Musher, 1961). Reduction of *d*- or *l*-IXu, as shown in Figure 8, for example, will give a product with the 10-methyl and 2-hydroxyl groups *cis* to each other and, although the initial product may be represented as an axial alcohol, ring flip will

[8] The *cis*-2-decalones are pictured in the steroid conformation which Djerassi and Marshall (1958) considered on the basis of optical rotatory dispersion studies to be the most stable form. Recent nmr studies (Elliott and Robinson, 1965) indicate that the nonsteroid conformation may be slightly favored. Since the relative stability of the two forms depends on the skew interaction of two methylene groups being worse than that of a methylene group and a carbonyl oxygen, it may be suggested that in aqueous medium, as in the present study, the steroid conformation would be favored due to solvation of the carbonyl. Regardless of the precise concentration of the two forms in solution, the much more rapid reduction of the *d* than the *l* compound and the effect on rate of the angular 10-methyl group are consistent only with enzymatic reduction of the *cis*-2-decalones occurring in the steroid conformation. Further, in the nonsteroid conformation, a methylene group is present that corresponds to the unfavorable methyl position of 4-*t*-butylcyclohexanone (C-31, Figure 12) which would lead to a rate prediction for the *cis*-2-decalones far below that which was actually observed.

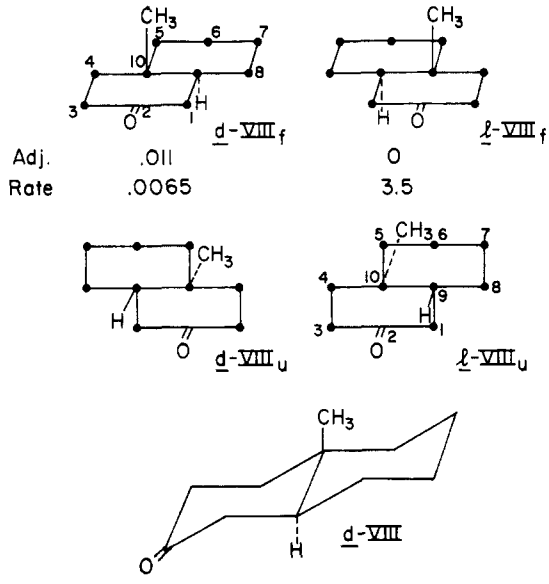


FIGURE 6: Reduction positions and adjusted rates for *d*- and *l*-10-methyl-*trans*-2-decalone.

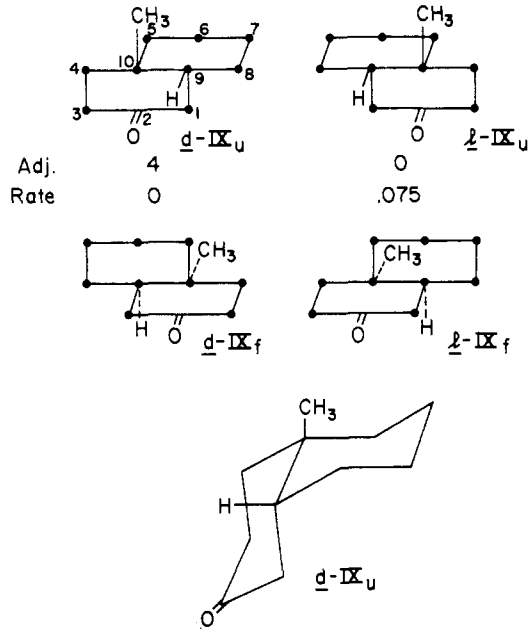


FIGURE 7: Reduction positions and adjusted rates for *d*- and *l*-10-methyl-*cis*-2-decalone.

immediately occur to the more stable conformation with the alcohol equatorial (IXu → Xa → Xb). Reduction in the positions *d*- or *l*-IXf, however, lead directly to the stable equatorial hydroxy compounds with hydroxyl and 10-methyl groups *trans* (*trans*-alcohol, Xc). Comparison of the four *cis*-bicyclic positions (Figure 7) with the four 3-methylcyclohexanone positions (Figure 5) reveals that only *d*-IXu could be expected to undergo rapid reduction since its first ring

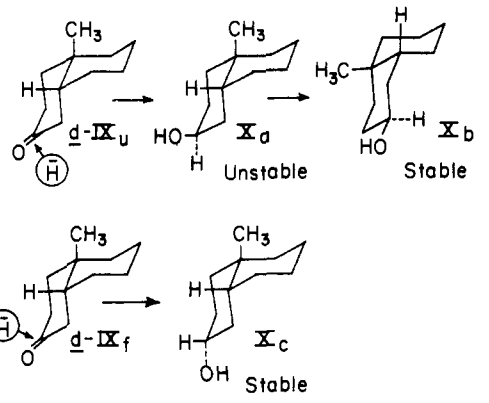


FIGURE 8: Conformations of 10-methyl-*cis*-2-decalols.

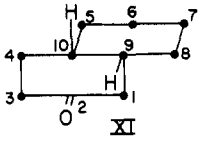


FIGURE 9: *d-cis*-2-Decalone.

upright and 3-alkyl substituent on the right correspond to (−) VIIu. Selective reduction of *d*-IXu and exclusive formation of the *cis*-alcohol Xb, as actually observed, follow logically then from the 3-methylcyclohexanone effect.

The very slow rate of reduction of the *l* enantiomer (*l*-IXf) stems primarily from a 3-methylene substituent located as in (+) VIIf or (+) VIIu. The favoring of f over u, as shown by the exclusive formation of *trans*-alcohol, is predictable on the basis of a 3-methyl inhibition effect which is ten times more marked in (+) VIIu than in (+) VIIf. In addition, both bicyclic compounds may be considered as derivatives of 4-methylcyclohexanone which predicts a further favoring of *l*-IXf over *l*-IXu since the angular methyl substituent, which corresponds to a 4-methyl group in III (Figure 2), occupies the unfavorable upright position in *l*-IXu.

dl-cis-2-Decalone. The extremely high reduction rate of *dl-cis*-2-decalone (Figure 9) (XI pictures the *d* compound), which was 15% of cyclohexanone, merits further comment and analysis. Since *dl*-IX and *dl*-XI differ by only the angular methyl substituent, their 6.8-fold difference in rate (Table III) measures the inhibition effect of that sole methyl group, and although the effect is somewhat higher than that calculated in the 4-methylcyclohexanone case it is of the same order of magnitude. Although the individual enantiomers of *dl*-XI have not been studied it may be anticipated that the initial velocity measures primarily the reduction of the *d* compound, as in the 10-methyl-*cis*-decalones (IX). Since the rate is almost directly proportional to concentration at the low substrate concentrations utilized, the comparative velocity of the pure *d* isomer should be about 30% of cyclohexanone at

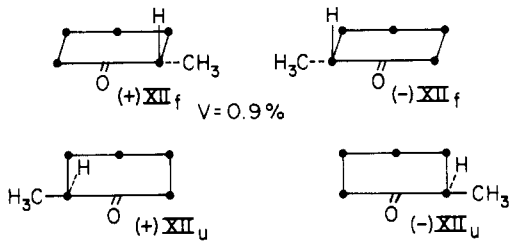


FIGURE 10: Possible reduction positions for (+)- and for (−)-2-methylcyclohexanone.

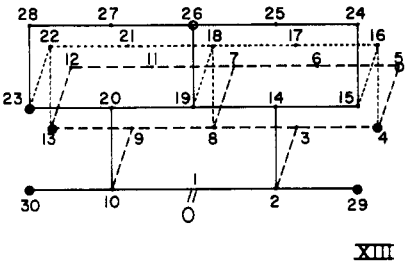


FIGURE 11: Composite structure of satisfactory and unsatisfactory substrates in front view. Solid black circles indicate positions of severe inhibition and, with the exception of 29, represent either methyl or methylene groups. C-29 represents a severe inhibition position only when occupied by CH_3. The large open circles indicate positions of moderate inhibition.

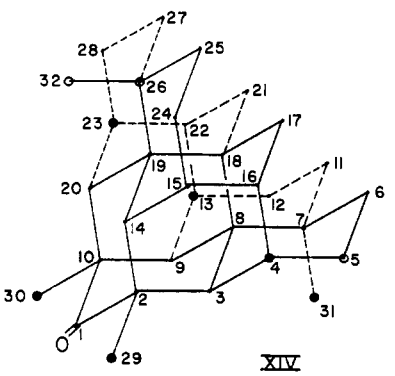


FIGURE 12: Composite structure of satisfactory and unsatisfactory substrates in side view.

equivalent molar concentrations. Taking (−)-3-methylcyclohexanone [position (−) VIIu, Figure 5] as a standard with an adjusted rate of 102%, it may be seen that C-5, -6, and -7 reduce the rate of reduction of XI by a factor of only 3.4 times. In the following section the individual rate inhibition effects are analyzed in greater detail.

2-Methylcyclohexanones. The oxidative and reductive studies of Prelog (1963, 1964) with 2-methylcyclohexanones and -cyclohexanols demonstrated greater than a 100-fold reduction in reaction rate due to an equatorial 2-methyl group. This effect was noted when the methyl group was positioned on either the right- or left-hand side and when the cyclohexanone ring was in either the flat or upright position. Our reductive studies, which exhibited an over-all rate of 0.9 for the (±) compound (XII, Figure 10), are thus fully confirmatory although individual enantiomers were not studied.

Composite and Individual Rate Inhibition Effects. A single composite structure may be readily assembled from Dreiding models that encompasses all positions of the 2-, 3-, and 4-alkylcyclohexanones as well as the bicyclic compounds when the rings are in the chair form. This composite is shown in front view (XIII, Figure 11) and also schematically represented in side view (XIV, Figure 12). In order to present a uniform rate picture, each position, which represents either a methyl or methylene group, has been numbered, but these numbers do not correspond to those in the individual formulas (I–XII). The *l*-10-methyl-*trans*-2-decalone (*l*-VIIIu), for example, is traced by numbers 1, 2, 14, 15, 24, 25, 26, 19, 20, and 10, with 18 representing the angular methyl group; the *d*-*cis*-2-decalone (*d*-IXu) is traced by 1, 2, 14, 15, 16, 17, 18, 19, 20, and 10, with 26 as the angular methyl substituent. The *l*-*trans*-2-decalone in the flat position (*l*-VIIIf) is traced by 1, 2, 3, 8, 7, 11, 12, 13, 9, and 10, with 18 again representing the angular methyl group. Groups 29 and 30 represent the two possible positions for an equatorial 2α-methyl group in (±)-2-methylcyclohexanone while 31 and 32 (XIV) represent the methyl group R of *t*-butylcyclohexanone, as shown in Figure 4 by V and VI, respectively. Although not shown on Figure 11, 31 projects down from 7 and 32 projects out from 26. Numbers 7 and 26 represent the two positions for the 4-methyl group of 4-methylcyclohexanone, but they also represent the angular methyl group for the *cis*-bicyclic compounds in their two possible positions as well as ring methylene groups of the *d*- and *l*-*trans*-2-decalones. Further, 7 and 26 constitute part of the alkyl group of the isopropyl- and *t*-butylcyclohexanones, as do the pairs 6–11 and 25–27. The four numbers 6, 11, 25, and 27 also each represent one of the methylene groups in the four possible positions for the *d*- and *l*-*trans*-2-decalones. Numbers 4, 13, 15, and 23 represent the four positions for the 3-methyl group in (±)-3-methylcyclohexanone and as noted earlier each constitutes a methylene group in one position of a *cis*- and of a *trans*-2-decalone. Number 18 constitutes a methylene group in all possible positions of a *cis*-2-decalone and also represents the angular methyl group in each position of the 10-methyl-*trans*-2-decalones. Numbers 5, 12, 24, and 28 appear, respectively, in the *trans*-2-decalone positions *d*-VIIIf, *l*-VIIIf, *l*-VIIIu, and *d*-VIIIu, while either 16 or 22 appears in the four *cis*-2-decalone positions but in no other substrate.

From formulas XIII and XIV, in particular the latter, it may be seen that each carbon position (understood to represent either methyl or methylene) may be classified as an outer carbon atom (right or left), an inner carbon atom (right, left, or center), or as a peripheral

TABLE VI: Estimated Inhibition Factors of Specific Carbon Atoms.

Carbon Atoms Outer (right)	Inhibn. Factor	Carbon Atoms Peripheral	Inhibn. Factor
4	110	*31*	108
5	13	*32*	2.9
15	2-fold stimulation		
16	1.7	Inner (right)	
24	2.6		
29	>100	6	1.4
		17	1.7
Outer (left)		25	1.3
12	Cannot calculate	Inner (left)	
13	>100		
22	Cannot calculate	11	1.4
23	1100	21	Cannot calculate
28	None	27	1.3
30	>100		
		Inner (center)	
		7	1.7
		18	1.2
		26	6.8

carbon atom (31 and 32). All substituents which are not encountered in the basic skeleton of cyclohexanone in the flat or upright position are classified in Table VI in that manner. It is evident from XIV that parallel planes, each perpendicular to the plane defined by 2, 3, 9, and 10, may be passed through the carbon atoms listed in each group; the plane passing through the inner carbon atoms (center) also passes through the peripheral carbon atoms.

An inhibition factor, which is a measure of the unfavorable steric interaction produced in the ternary complex (enzyme–$NADH_2$–ketone) by a particular substituent on a cyclohexanone ring, may be calculated for almost all of the positions listed in Table VI. With the probable exception of the 2-methylcyclohexanone effect (C-29), which is discussed in detail later in this paper, the inhibition effect of a given substituent appears to be relatively constant regardless of the substrate so that methyl and methylene groups may be treated in an identical fashion.

Inhibition factors for 4, 13, 23, and 15 have already been treated in the 3-methylcyclohexanone discussion and shown to be 110-, 1100-, >100-, and 2-fold stimulation when compared with cyclohexanone as standard. The inhibition factors of >100 for carbons 29 and 30 follow from the 2-methylcyclohexanone and 2-methylcyclohexanol rates. Inhibitions of 108- and 2.9-fold for carbons 31 and 32 came from the comparison of isopropyl- and *t*-butylcyclohexanone. Comparison of 4-methylcyclohexanone with cyclohexanone gave inhibitions of 1.7 for C-7 and 4.2 for C-26. However, the value of 6.8 for C-26, which came from comparison of *dl-cis*-2-decalone and *dl-cis*-10-methyl-2-decalone, is taken as the more accurate value since the value derived from 4-methylcyclohexanone involved the assumption that cyclohexanone reduces equally in the flat and upright positions. C-18 was shown to inhibit by a factor of only 1.2 in the comparison of *dl-trans*-2-decalone and *dl*-10-methyl-*trans*-2-decalone. Comparison of the adjusted rates of 4-isopropylcyclohexanone and 4-methylcyclohexanone in the flat position gives an inhibition factor of 2.07 for C-6 and -11. For simplicity it is assumed that the effect of each carbon is identical, which gives an inhibition factor of 1.4 for each carbon. Similar treatment of the 4-methyl and 4-isopropyl compounds in the upright position gives an inhibition factor of 1.3 each for C-25 and -27.

The factor for C-24 comes from the comparison of *l-trans*-10-methyl-2-decalone in the upright position (*l*-VIIIu) with (−)-3-methylcyclohexanone in the upright position [(−) VIIu]. The 29.2-fold difference in adjusted rates must come from C-24, -25, -26, and -18. From the values already calculated the inhibition due to 25, 26, and 18 is equal to $1.33 \times 6.84 \times 1.22 = 11.1$, which leaves an inhibition factor of 2.6 for C-24.

In a similar fashion the values for C-16 and -17 stem from a comparison of *d-cis*-10-methyl-2-decalone (*d*-IXu) with (−)-3-methylcyclohexanone [(−) VIIu]. The adjusted rate differences of 25-fold due to C-16, -17, -18, and -26 leaves an inhibition factor of 3.0 due to 16 plus 17. Arbitrarily, an equivalent inhibition factor of 1.7 is assigned to each carbon.

A highly tentative inhibition factor may be assigned to C-5 by comparing the adjusted rate of *d-trans*-10-methyl-2-decalone in the flat position (*d*-VIIIf) with 4-methylcyclohexanone (IIIf). Taking 0.0115 as the adjusted rate for *d*-VIIIf, and accounting for C-4, -6, and -18, a factor of 13 is left for C-5. The accuracy of

this figure is limited by the relatively large error in the measurement of very slow rates. By taking (+)-3-methylcyclohexanone [(+) VIIIf] as a standard and correcting for C-6, -7, and -18, an identical value may be calculated for C-5. Although the precise magnitude of the inhibition effect of this carbon is clearly open to question, the point to be noted is that there is a significant inhibition, but not nearly so great as C-4.

Within the limitations pointed out in the C-5 calculations, it may be shown that C-28 has little or no inhibitory effect upon the rate of reduction. The *d*-10-methyl-*trans*-2-decalone adjusted rate in the upright position (*d*-VIIIu) is 0.006. Taking 4-methylcyclohexanone in its upright position (IIIu) as standard and correcting for C-18, -23, and -27, a rate of 0.007 would be predicted for *d*-VIIIu if there is no effect for C-28. If (+)-3-methylcyclohexanone upright [(+) VIIu] is taken as standard and correction made for C-18, -26, and -27, a rate of 0.004 would be anticipated for *d*-VIIIu when C-28 has no effect.

The effects of C-12, -21, and -22 cannot be calculated since none of the substrates in the positions in which they underwent reduction had methyl or methylene groups corresponding to those positions.

One additional example may be cited to demonstrate this quantitative approach to inhibition factors. The reduction rate of the *l*-10-methyl-*cis*-2-decalone (*l*-IXf), corrected for the 1.9% of *d* enantiomer, is 0.075. This particular substrate has not been previously used to calculate any of the inhibition factors. By taking (+)-3-methylcyclohexanone flat [(+) VIIf] as standard with its adjusted rate of 0.46 and applying the inhibition factors for C-16, -17, -18, and -7, a predicted rate of reduction of 0.078 is obtained for *l*-IXf. In view of the measurement errors involved, the remarkable agreement of predicted and observed rate must contain a substantial element of chance, but the agreement of values does indicate the validity of this approach.

Carbon positions with an inhibition factor of greater than 100 (4, 13, 23, 29, 30, and 31) are represented by a large solid circle in XIII and XIV, while those with intermediate factors (2.9–13) are shown as a large open circle (5, 26, and 32). It is evident that there is a general area of inhibition on the right, an area on the left, and one or more in center positions represented by C-26, -31, and -32. Anticipating the discussion to follow, we attribute the inhibition on the right to interference with binding of the substrate at a hydrophobic bonding site while that on the left is attributed to steric interaction with the coenzyme or its bonding site.

The apparent constancy of inhibition factors in the various derivatives of cyclohexanone, including the bicyclic compounds, indicates that there is little or no conformational adjustment of the enzyme to accommodate different substrates. While our data is insufficient to take issue with the induced-fit hypothesis of Koshland (1958), it must be noted that the enzyme may be treated as a rigid structure in the ternary complex of enzyme–coenzyme–cyclohexanone derivative. This in no way infers that a conformational change of enzyme does not occur upon binding of the coenzyme and/or substrate, or even that the conformation of the enzyme must be the same in both an aldehyde and ketone ternary complex; only that the enzyme conformation is similar with all cyclohexanone derivatives.

Up to this point the stereospecificity of reduction has been considered only in terms of steric interference due to one or more substituents on a cyclohexanone ring and it has been shown that a single methyl or methylene group at an unfavorable position can determine which of two enantiomers will undergo reaction, or which of two possible products will be formed from a single substance. However, it is quite apparent that with aldehydes and primary alcohols as substrate another type of stereospecificity pertains. In contrast to derivatives of cyclohexanone and cyclohexanol which have a carbon atom at positions 10 and 2 (XIII and XIV), the aldehydes and primary alcohols may have a carbon atom occupying either position 2 or 10, but obviously not both at the same time; if position 2 is occupied, the substrate may be considered to be bound with the hydrophobic portion of the molecule to the right of the carbonyl group while if position 10 is occupied, the hydrophobic portion lies to the left of the carbonyl. If binding on one side is markedly favored over the other, the enzyme should be able to discriminate between the two hydrogen atoms or between a hydrogen and deuterium atom on the hydroxyl-bearing carbon atom of a primary alcohol. This discrimination has been demonstrated, for example, with yeast alcohol dehydrogenase, where deuterioethanol which was stereospecifically prepared by the reduction of acetaldehyde with deuterio-$NADH_2$ underwent a complete loss of deuterium when the alcohol substrate was reoxidized with NAD and the same enzyme (Loewus *et al.*, 1953; Levy *et al.*, 1957). With liver ADH the same type of pattern was observed with geraniol [1-R(1-^{3}H)-geraniol] that was stereospecifically labeled with tritium by chemical means (Donninger and Ryback, 1964). Enzymatic dehydrogenation to the aldehyde geranial resulted in an 80% loss of tritium. Since the chemical labeling was only about 80% specific, the enzymatic process is believed to be completely specific. These interconversions are shown in Figure 13 with (H*) representing deuterium or tritium. Reduction of an aldehyde XV with heavy-atom-labeled $NADH_2$ leads to the alcohol XVIr when the aldehyde is so positioned that the alkyl group lies on the right as in XVr; reduction of the same aldehyde positioned so that the alkyl group lies on the left (XVl) leads to the alcohol XVIIl. Alcohols XVIr and XVIIl can be enzymatically differentiated since reoxidation of the former leads to a loss of the heavy hydrogen label only when the alkyl group lies on the right, while the alkyl group of XVIIl must lie on the left for heavy hydrogen loss to occur. The tritiated geraniol work (Donninger and Ryback, 1964) demonstrates that with liver ADH, the alkyl group must lie on the right as in XVr and XVIr. It appears reasonable to conclude then that with liver ADH all aldehydes and the corresponding alcohols occupy this position in the ternary complex of enzyme–coenzyme–substrate.

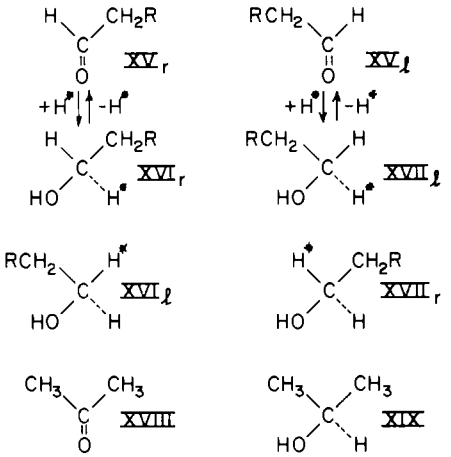


FIGURE 13: Oxidation and reduction positions for primary alcohols and for aldehydes demonstrating the requirements for stereospecific deuterium or tritium transfer.

In theory, this preference for the right side over the left side could be due to preferential binding of the substrate on that side by favorable hydrophobic interactions with the enzyme, or the preference could be due to greater steric hindrance on the left than on the right. Preference could also be determined by a combination of these two factors. It should be noted at this point that if, with a given enzyme, a precise positioning with respect to the pyridine nucleotide is required for the reacting carbonyl or hydroxyl group of the substrate, then greater steric hindrance to the placement of the alkyl group on one side than the other is sufficient to explain stereospecificity. Under these conditions it is not necessary to invoke three points of attachment for the substrate, as, for example, in the Ogston theory (1948), in fact stereospecificity could be exhibited with no formal points of substrate–enzyme attachment. We do not propose, however, that this is the case.

It is our opinion that there is a substrate–enzyme hydrophobic attachment site, which, on the basis of the geraniol example, lies on the right. The methyl groups of ethanol and acetaldehyde are then represented by C-2 in XIII and XIV. The fact that methanol and formaldehyde lack an alkyl group and are essentially inactive with liver ADH is explicable most readily on the basis of a hydrophobic bonding of the alkyl group of ethanol and acetaldehyde. Also, the fact that compounds with increased chain length up to *n*-butyl alcohol and *n*-butyraldehyde as well as certain aromatic aldehydes and alcohol are more efficient substrates than ethanol and acetaldehyde (Sund and Theorell, 1963) indicates that these substrates undergo a greater degree of favorable hydrophobic bonding with the enzyme.

It appears equally clear that, apart from the alkylcyclohexanone effects which have already been described in detail, certain subtle steric factors may determine whether or not a substance serves as a satisfactory substrate for liver ADH. The cases of cyclopentanone–cyclopentanol and acetone–isopropyl alcohol may be cited. All four of these substances are symmetrically substituted about the carbonyl group or about the carbon bearing the hydroxyl group, and none serve as a satisfactory substrate for liver ADH despite the obvious similarity to cyclohexanone. Acetone (XVIII) and isopropyl alcohol (XIX) (Figure 13) may also be considered as simple derivatives of the "natural" substrates acetaldehyde and ethanol except that they bear an additional methyl substituent on the left-hand side when XVr and XVIr represent the natural substrates in the ternary complex. This position on the left is occupied by a fixed CH_2 group in the case of cyclohexanone and cyclohexanol, which suggests the possibility that a rotating methyl group on the right, as in ethanol and acetaldehyde, may be "frozen" by the enzyme, while the same group on the left-hand side cannot be fixed in a manner that minimizes steric interactions in the ternary complex. Alternately, the difference between acetaldehyde and acetone may be explained on the basis of favored conformations of the two, since considerable evidence indicates that in acetaldehyde the conformation with minimum energy is one in which a hydrogen eclipses the carbonyl oxygen (Allinger *et al.*, 1964). In this position the hydrogen atoms occupy precisely the same position as the α-methylene hydrogens in cyclohexanone (shown in Newman projection).

In contrast the lowest energy conformation of acetone appears to be one wherein the carbonyl group is staggered with respect to a pair of hydrogen atoms on each methyl group so that a hydrogen atom projects below the plane of the carbonyl.

Taking the positions of the hydrogen atoms in the α-methylene groups of the cyclohexanone derivatives as permitted positions consistent with high enzymatic activity, it follows that forbidden positions in the methyl group are ones in which a hydrogen atom extends below the plane of the carbonyl group. We attribute the inactivity of isopropyl alcohol and acetone, then, to a C–H bond of a methyl group extending below the plane of the carbonyl. From the discussion above, the methyl group on the left may be chosen as the offender if the enzyme can "fix" a methyl on the right, or both methyl groups of acetone could be offenders if the conformations of acetaldehyde and acetone in the ternary complex are those represented by the Newman projections. In light of this discussion the inactivity of cyclopentanone (XX) (Figure 14) and cyclopentanol are readily explicable since in a five-membered ring the methylenic hydrogen atoms are neither equatorial nor axial but

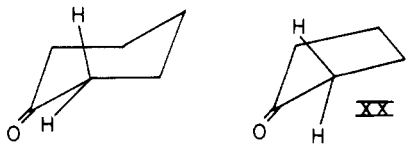


FIGURE 14: Carbon–hydrogen bond angle differences in cyclohexanone and cyclopentanone derivatives.

project above and below the plane of the carbonyl group at approximately equal angles.

Based on the compounds that are satisfactory and unsatisfactory substrates certain additional conclusions may be made with respect to the hydrophobic binding site on the right. A methyl group placed at C-2 (XIII and XIV) constitutes a binding site for ethanol and acetaldehyde. Presumably the methylene group of other aliphatic aldehydes and primary alcohols located at that same position serves an identical function. Aromatic aldehydes such as furfural and benzaldehyde, both of which (as well as the corresponding alcohols) are excellent substrates, do not have any hydrogen atoms attached to C-2; therefore a C–H bond is not necessary for hydrophobic bonding at that position. Carbon C-4 and -29 (XIII and XIV), each of which decrease the rate by a factor of more than 100, are close to the binding site at C-2, and it may be tentatively concluded that they exert their inhibitory influence by interfering with enzyme binding of substrate at that site. It has been shown that the inhibitory influence at C-4 is of the same order of magnitude when that position is occupied by a methyl group (3-methylcyclohexanone) or by a methylene group (as in the *cis*- and *trans*-2-decalones). With respect to C-29, a methyl group has been shown to inhibit reduction by a factor of over 100, but the case is not so clear with respect to a methylene group. A number of examples of *trans*-1-decalol and -1-decalone derivatives cited by Prelog (1963, 1964) show that these compounds cannot serve as substrates with liver ADH. In one of the two possible positions for these compounds (*i.e.*, either flat or upright) a methylene group is present at C-2. However, when C-2 is occupied, two additional methylene groups to the right of C-2 are present in the upright position and three additional methylenes (including one at C-4) are present in the flat position. The assertion that the inactivity of these substrates stems from the equatorial substituent at C-2 completely ignores the effect of the other methylene groups. Two *cis*-1-decalols (Figure 15) have been reported (Prelog, 1964) to undergo oxidation at 8 and 0.4% of the rate of cyclohexanol, respectively. The structure XXIa, with hydroxyl group axial, has been assigned to the faster compound and the structure XXII, with hydroxyl equatorial, has been assigned to the slower substance. In both XXIa and XXII, a methylene group is present at the position corresponding to C-4 in XIII and XIV, a position shown to decrease reduction rates by a factor of over 100. Therefore, the structure XXIa is inconsistent with its relatively high oxidation

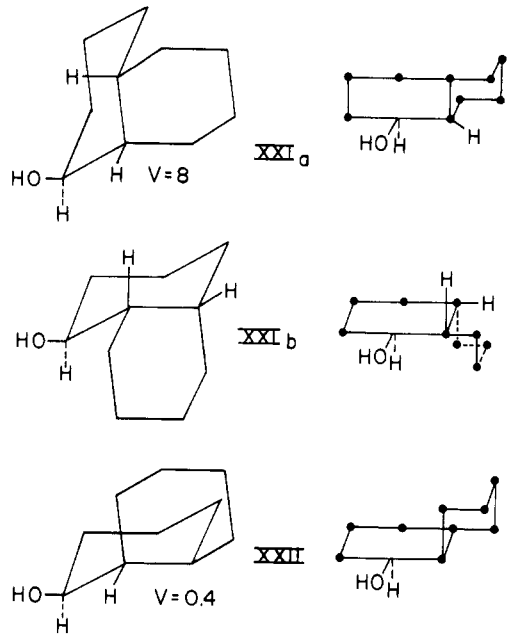


FIGURE 15: Conformations of *cis*-1-decalols.

rate while the rate of XXII is consistent with its structure, which is not questioned. As noted earlier in the paper, *cis*-decalols with an axial hydroxyl group are conformationally unstable since they may undergo ring flip to a thermodynamically more stable structure with the hydroxyl group equatorial (Musher, 1961; Feltkamp *et al.*, 1965). The more stable conformation for XXI, then, is actually represented by XXIb rather than XXIa, and in this form there is no methylene corresponding to C-4 in XIII; instead, ring B projects below ring A. In this position, however, there is a methylene group corresponding to C-29 in XIII, and the question now arises whether a methylene group at C-29 can be consistent with relatively high enzymatic activity (*e.g.*, 8% of cyclohexanol) while a methyl group as in 2-methylcyclohexanone or 2-methylcyclohexanol is not. If it is assumed that, in the ternary complex of enzyme–$NADH_2$–aldehyde, the excellent substrate benzaldehyde lies in its most stable form with the carbonyl group and the benzene ring planar, then the question may be answered in the affirmative. Superimposition of the carbonyl groups of benzaldehyde and cyclohexanone, as shown in XXIII (Figure 16), results in one of the C–H groups of benzaldehyde lying practically on top of C-29 (the lower solid dot), while the plane of the ring is tipped considerably above C-4 which is represented by the upper dot. Although other positions for benzaldehyde are possible by moving the ring and the carbonyl function out of plane (*e.g.*, XXIV) and these positions do not place C–H groups from the aromatic ring in the vicinity of either C-4 or -29, these positions seem unlikely in terms of the large loss of resonance energy that would result from such out-of-plane conformations. It is our opinion then that XXIb rather than XXIa represents the conformer that reacts with the enzyme.

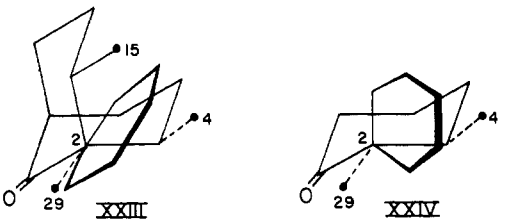


FIGURE 16: Positioning of benzaldehyde relative to cyclohexanone during enzymatic reduction. The darker ring represents the aromatic ring of benzaldehyde; the position of the carbonyl function is identical for both substrates.

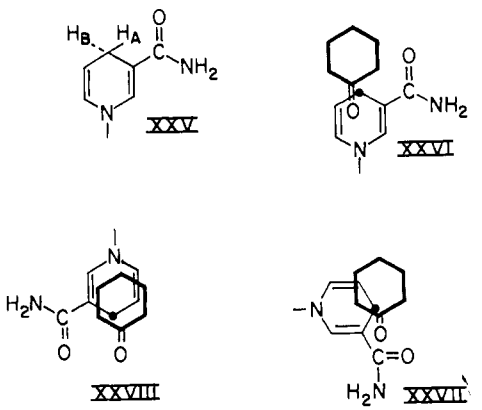


FIGURE 17: Positioning of A-$NADH_2$ relative to substrate. The black dot represents the A hydrogen of $NADH_2$ and is understood to be beneath the carbonyl group. The planes of the dihydropyridine and cyclohexanone rings need not be parallel to each other.

Since substrates such as butyraldehyde, benzaldehyde, and furfuraldehyde are reduced faster than acetaldehyde at low but corresponding substrate concentrations, it follows that there must be additional hydrophobic bonding sites besides C-2 (XIII and XIV). The fact that C-15 occupies an outside position and yet appears to actually increase the rate of reduction leads to the highly tentative suggestion that a methyl or methylene group at C-15 may serve as an additional hydrophobic bonding site. A segment of enzyme passing between positions 2 and 15 would pass across a significant portion of the top of the benzene ring of benzaldehyde when that substrate is located in its thermodynamically most favorable conformation (XXIII) and thus account for the high activity of that compound as well. An equally tentative location for the skeleton of the substrate butyraldehyde could then be represented by C-1, -2, -14, and -15.

Finally, the question of the location of NAD and $NADH_2$ relative to the substrate may be approached. A-$NADH_2$ (XXV, Figure 17) has been considered (Prelog, 1963, 1964) to be located directly in front of the substrate so that the A hydrogen, which undergoes transfer (Cornforth *et al.*, 1962), lies directly under the substrate carbonyl and the carboxamide group lies to the right (XXVI). Justification for locating $NADH_2$ in this manner was the minimization of nonbonded interactions and the assumption that a substrate bearing a substituent at C-29 (XIII) is of necessity a poor substrate due to unfavorable steric interactions of position 29 with the carboxamide group of the pyridine nucleotide. Evidence has been presented above that C-29 can, in fact, be occupied in a satisfactory substrate which appears to remove this basic premise. We prefer instead, for liver ADH, the position XXVII with a portion of $NADH_2$ under the substrate but at essentially a right angle to the cyclohexanone ring. The black dot represents the A hydrogen of $NADH_2$, and the planes of the dihydronicotinamide and cyclohexanone rings need not be parallel. In fact, if the 1,4-dihydronicotinamide ring is planar, as suggested by the nmr studies of Meyer *et al.* (1962), the A and B hydrogens of $NADH_2$ will be staggered relative to the nicotinamide ring rather than in true axial–equatorial positions. With this conformation hydride transfer could readily occur, or might even be more favorable, when the nicotinamide ring is tipped relative to the substrate so that the $NADH_2$ nitrogen at position 1 is considerably below the carbon atom at position 4. If, however, there is a strict requirement for axial (perpendicular) hydride transfer at the transition state, then the two rings in question must be essentially parallel. The carboxamide group in position XXVII does not interact with the substrate and, more important, it is not in a position where it can sterically interact with a hydrophobic bonding site on the right-hand side of the substrate. In contrast, the carboxamide group lying on the right as in XXVI would probably interfere with such a bonding site and would not appear to permit a satisfactory "fit" of substrates such as benzaldehyde and furfural, and certainly not the *cis*-1-decalol represented by XXIb in Figure 15. Further, it should be noted that NAD, chemically modified by a bulky isopropyl substituent on the nitrogen of the carboxamide group, acts as a satisfactory coenzyme (Anderson and Kaplan, 1959). This would not appear to be possible if the carboxamide lies to the right as in XXVI. The marked decrease in substrate activity due to substituents on the outer left side (C-13, -23, and -30, XIII and XIV) we then attribute to steric interference of the substrate with the coenzyme or with a portion of enzyme binding the coenzyme. The inactivity of substrates such as acetone and cyclopentanone which have a hydrogen atom at C-10 projecting below the plane of the carbonyl is explicable, at least in part, on the same grounds, and even the reduced activity of cyclohexanone relative to aldehydes may be due to steric interaction of the hydrogens at C-9 and -10 (XIII and XIV) with the pyridine nucleotides. Position XXVIII for $NADH_2$, with the dihydronicotinamide ring directly under the cyclohexanone ring, appears quite unfavorable due to nonbonded interactions with axial hydrogens of cyclohexanone, in particular if the nicotinamide and cyclo-

hexanone rings are parallel. If the nicotinamide ring is sufficiently tipped, however, as discussed above, then XXVIII would constitute a satisfactory arrangement for substrate and coenzyme, although we favor XXVII.

The marked inhibition by C-31, which projects below the substrate in a relatively isolated position, would not appear to reflect an interference with either the coenzyme binding site on the left or the substrate binding site on the right but might instead result from a nonfunctional segment of the enzyme passing beneath the plane of the substrate and connecting the two binding sites.

Acknowledgments

The authors should like to acknowledge the able technical assistance of Mr. Harold Lawrence, Jr., and wish to thank Mr. Sanford L. Patashnik for the ORD determinations.

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