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The *in vitro* and *in vivo* metabolism of optically active methylcyclohexanols and methylcyclohexanones

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The *in vitro* reduction and oxidation of the isomeric methylcyclohexanones and methylcyclohexanols by horse liver alcohol dehydrogenase, in the presence of $NADH_2$ or NAD has been studied. The enzymic reactions closely resemble the corresponding *in vivo* metabolic conversions and support the view that the oxido-reductase in the animal body is similar to liver alcohol dehydrogenase. The findings are discussed in terms of the conformations of the alcohols and ketones and a correlation of the observed rates, K_m values, and the steric specificity supports the view that the *in vivo* inversion of the thermodynamically less stable alcohols (±)-*cis*-2-, (±)-*trans*-3-, and *cis*-4-methylcyclohexanol could occur through a ketone intermediate.

From the studies of Prelog (1959), and Graves, Clark & Ringold (1965) it appears that an oxido-reductase system is responsible for the biological oxidation and reduction of alicyclic alcohols and ketones. The identity of the enzyme, however, has not yet been clearly established, although Tao & Elliott (1962) suggested that LADH might be responsible, though Prelog (1964) considered that this could not be the enzyme as it would not reduce the *trans*-decalin-1,4-diones.

Views vary about the stereochemical course of the reaction. Prelog (1959 & 1964) proposed a "head-to-tail" orientation for the substrate relative to the coenzyme in the transition state, Tao & Elliott (1962) suggested a "face-to-face" orientation, Graves & others (1965) have proposed two orientations—"flat and upright"—and Cheo, Elliott & Tao (1967) have also suggested that there are two possible orientations which they describe as "face-to-face" and "perpendicular".

The present investigation attempts to clarify these two problems through a systematic comparison of the biological and enzymic transformations of the enantiomeric methylcyclohexanones and methylcyclohexanols. In addition the opportunity has been taken to present a simple stereochemical model of the reaction mechanism which appears to have a reasonably good predictive value.

MATERIALS

Liver alcohol dehydrogenase (LADH—Alcohol: NAD oxido-reductase [E.C. No. I.I.I.I.]), twice recrystallized and prepared by the method of Bonnichsen & Brink (1955) was purchased from Worthington Biochemical Corporation, Freehold, N.J. Nicotinamide adenine dinucleotide (NAD) and reduced nicotinamide adenine dinucleotide ($NADH_2$) were obtained from Sigma Chemical Company, St. Louis.

Tritiated nicitonamide adenine dinucleotide NAD(T) was prepared according to San Pietro (1955). All derivatives of methylcyclohexane were purified by preparative

gas-liquid chromatography and distillation under reduced pressure and were characterized by thin-layer and gas-liquid chromatography; their retention time, Rf values, refractive indices and optical rotations at 589 and 305 or 309 nm are given in Table 1.

(±)-2-, (±)-3-, and 4-Methylcyclohexanone were obtained commercially (Eastman Kodak Ltd., Rochester). (+)-2-, (−)-2-, and (−)-3-Methylcyclohexanone were obtained by chromic acid oxidation of (+)-*trans*-2, (+)-*cis*-2-, and (+)-*cis*-3-methylcyclohexanol respectively (Ohloff, Osiecki & Djerassi, 1962). (+)-3-Methylcyclohexanone was obtained from (+)-pulegone by refluxing with 2·5N HCl.

The optically inactive isomeric methylcyclohexanols (±)-*cis*-, and (±)-*trans*-2-methylcyclohexanol (±)-*cis*,- and (±)-*trans*-3-methylcyclohexanol, and *cis*-, and *trans*-4-methylcyclohexanol were separated from commercial (Eastman Kodak Ltd) *cis*/*trans* mixture of the 2-, 3-, and 4-methylcyclohexanols respectively, by preparative gas-liquid chromatography. The optically active isomeric methylcyclohexanols were prepared biologically by feeding the appropriate ketone to rabbits and isolating the corresponding glucuronides from the 24 h urine by the basic lead acetate method of Kamil, Smith & Williams (1951). The glucuronide or its triacetylmethyl ester was then hydrolysed with N HCl and the aglycone was recovered by steam distillation and extraction of the distillate with ether. For the preparation of (+)-*cis*-, and (+)-*trans*-2-methylcyclohexanol, (±)-2-methyl-cyclohexanone was fed. (−)-*cis*, and (−)-*trans*-3-Methylcyclohexanol were obtained by feeding (+)-3-methylcyclohexanone. (+)-*cis*-3-Methylcyclohexanol was prepared by diastereomeric resolution and hydrolysis of the triacetylmethyl ester of the glucuronide obtained after feeding (±)-3-methylcyclohexanone.

METHODS AND RESULTS

Thin-layer chromatography

Plates were prepared as described by Elliott, Robertson & Williams (1966). For the separation of the 3-, and 4-methylcyclohexanones and methylcyclohexanols, solvent system 1 (light petroleum b.p. 50–70°–ethyl acetate, 9 : 1 v/v) was used. For the separation of the 2-methylcyclohexanones and methylcyclohexanols, solvent system 2 (light petroleum b.p. 50–70°–ethyl acetate, 8 : 2 v/v) was used. Alcohols were located with the phosphomolybdic acid spray of Kritchevsky & Kirk (1952), and ketones with Brady's reagent.

Gas-liquid chromatography, infrared spectroscopy and optical rotatory dispersion studies.

The instrumentation and conditions employed were as described by Cheo & others (1967), except where specifically indicated in the Tables.

Face specificity studies

A modification of the method of Krakow, Ludowieg & others (1963) was used. For alcohols, a control system comprising ethanol (0·1 ml), yeast alcohol dehydrogenase (YADH), (0·1 mg), NAD(T), (0·5 μmol) and sodium phosphate buffer (400 μmol, pH 9·6) in a total volume of 3 ml was used. After the first stage of the reaction was complete the mixture was boiled for 2 min, centrifuged and adjusted to pH 7 with NaH_2PO_4 (200 μmol) and sulphuric acid. Sodium pyruvate (12 μmol) and lactic dehydrogenase were then added, and the reaction followed spectrophotometrically at 340 nm.

The test system had the same composition and was treated in the same way as the control system except that 90 μmol of the appropriate methylcyclohexanol was used in place of ethanol and the YADH was replaced by LADH (200 mg). In both control and test systems, the nicotinamide was isolated according to Marcus, Vennesland & Stern (1958), dissolved in Panax TPP/3 solution and counted in a scintillation counter (Panax SC-LP). For ketones, the control system was prepared and treated as specified for alcohols. In the test system, NADT(H) was generated as described above for the alcohol control system except that, after centrifugation, acetaldehyde, which would interfere in the subsequent reaction, was removed by passing nitrogen through the mixture. In the second stage the appropriate ketone (90 μmol) and LADH (200 μg) were added and the subsequent procedure was as described for alcohols. Table 2 compares the tritium content of the nicotinamide obtained from the test and control systems for the various substrates, and indicates the stereospecificity of the transfer.

Kinetic studies

For the determination of Michaelis constants, reactions were followed spectrophotometrically on a Beckman DK-2A spectrophotometer at 340 nm in quartz cuvettes. Changes in absorbance were recorded continuously from within 10 s of the initiation of the reaction. Initial rates of change were measured and a steady state was assumed since the reactions were linear over the first 3-min. Ketones were dissolved in 0·003M NaH_2PO_4 buffer at pH 7·0, alcohols were dissolved in 0·1M

Table 1. *Physical properties, Michaelis constant and relative rates of oxidation or reduction of alcohols and ketones*

	*Retention time (min)	Rf	$[n]_D^{23}$	$[\alpha]_D^{23}$	$[\alpha]^{MeOH}$ †‡	K_m (molar)¶	Relative rates§
(±)-2-Methylcyclohexanone	3·1	0·85	1·4559	0	0	$1·79 \times 10^{-2}$	1·1
(+)-2-Methylcyclohexanone	3·1	0·85	1·4563	+14·5	+519	$1·03 \times 10^{-2}$	3·6
(−)-2-Methylcyclohexanone	3·1	0·85	1·4544	−13·9	−512	$2·12 \times 10^{-2}$	0·4
(±)-3-Methylcyclohexanone	4·1	0·46	1·4430	0	0	$1·44 \times 10^{-3}$	57·6
(+)-3-Methylcyclohexanone	4·1	0·46	1·4409	+14·3	+983	$1·19 \times 10^{-2}$	1·1
(−)-3-Methylcyclohexanone	4·1	0·46	1·4416	−13·6	−958	$1·48 \times 10^{-3}$	68·1
4-Methylcyclohexanone	4·1	0·50	1·4429	0	0	$1·88 \times 10^{-3}$	40·3
(±)-*cis*-2-Methylcyclohexanol	7·1	0·75	1·4621	0	0	$1·10 \times 10^{-2}$	12·7
(+)-*cis*-2-Methylcyclohexanol	7·1	0·75	1·4627	+8·9	−520	$1·55 \times 10^{-2}$	7·3
(±)-*trans*-2-Methylcyclohexanol	9·1	0·66	1·4580	0	0	$1·60 \times 10^{-2}$	15·5
(+)-*trans*-2-Methylcyclohexanol	9·1	0·66	1·4590	+22·4	+508	$1·21 \times 10^{-2}$	18·2
(±)-*cis*-3-Methylcyclohexanol	13·1	0·21	1·4550	0	0	$2·70 \times 10^{-3}$	35·4
(+)-*cis*-3-Methylcyclohexanol	13·1	0·21	1·4549	+6·0	−990	$1·75 \times 10^{-3}$	64·4
(−)-*cis*-3-Methylcyclohexanol	13·1	0·21	1·4559	−5·7	+970	$6·36 \times 10^{-3}$	22·5
(±)-*trans*-3-Methylcyclohexanol	9·8	0·27	1·4531	0	0	$1·56 \times 10^{-3}$	17·0
(−)-*trans*-3-Methylcyclohexanol	9·8	0·27	1·4540	−6·1	+968	$8·47 \times 10^{-3}$	12·4
cis-4-Methylcyclohexanol	9·6	0·30	—	0	0	$4·10 \times 10^{-3}$	36·9
trans-4-Methylcyclonexanol	12·9	0·21	—	0	0	$1·3 \times 10^{-3}$	52·1
Cyclohexanol						$5·52 \times 10^{-3}$	100·0
Cyclohexanone						$2·3 \times 10^{-3}$	100·0

* A 10% diglycerol/celite column was used at 80°C, flow rate 120 ml/min. Sample size was 0·1–0·5 μl of a 10% ethereal solution.

† 305 nm for the 2-ketone and 309 nm for the 3-ketone.

‡ Alcohols were oxidized by CrO_3 to the corresponding ketones before measurement.

¶ K_m values were obtained from Lineweaver and Burke plots.

§ Oxidation rates of ketones were compared with cyclohexanone at the same concentration, while rates of alcohol were compared with cyclohexanol.

Table 2. *Stereospecificity of hydrogen transfer*

Compound	Activities (counts 10^{-6}/100 s mg^{-1}) Test system (T)	Control system (C)	T/C × 100	Stereo specificity
(±)-2-Methylcyclohexanone	9·0	9·0	100	A
(±)-3-Methylcyclohexanone	1·1	1·1	100	A
4-Methylcyclohexanone	8·0	8·3	96	A
(±)-*cis*-2-Methylcyclohexanol	8·5	8·3	102	A
(±)-*trans*-2-Methylcyclohexanol	8·7	8·6	101	A
(±)-*cis*-3-Methylcyclohexanol	9·1	9·2	99	A
(±)-*trans*-3-Methylcyclohexanol	8·9	9·1	98	A
cis-4-Methylcyclohexanol	9·1	9·7	94	A
trans-4-Methylcyclohexanol	9·2	9·5	97	A

$Na_4P_2O_7$ buffer at pH 9·8. The substrate concentration was varied between $3·7 \times 10^{-4}$M and $7·8 \times 10^{-3}$M. Coenzyme concentrations were 1×10^{-4}M for $NADH_2$ and $0·95 \times 10^{-4}$M for NAD. The reactions were initiated by the addition of LADH, 35 μg in 0·2 ml of water. For the determination of relative rates of reduction the reactions were made as described for the K_m determinations except that the substrates were studied at a final concentration of 2×10^{-3}M. Table 1 lists the K_m values and relative rates of oxidation of the isomeric methylcyclohexanones and methylcyclohexanols.

Product formation from ketones

All reactions were studied at 24°. The ketone (16 or 50 mg) was dissolved in 0·003M NaH_2PO_4 buffer (200 ml), pH 7·0 to which was added $NADH_2$ (150 or 200 mg)

Table 3. *Product formation from ketones* in vitro

Compound	Amount of each reactant (mg) coenzyme	substrate	Unreacted ketone	$[\alpha]_D^{23}$	$[\alpha]^{MeOH}$*	Ratio *cis*/*trans*
(+)-2-Methylcyclohexanone	150	50	present	+22·4	+521	0/100
(−)-2-Methylcyclohexanone	150	50	present	+8·9	−512	100/0
(±)-2-Methylcyclohexanone	150	50	present	+8·9	−522	28/72
				+22·4	+520	
(±)-2-Methylcyclohexanone	150	16	trace	+8·9	—	45/55
				+22·4	—	
(±)-2-Methylcyclohexanone	200	16	undetected	+8·9	—	50/50
				+22·4	—	
(+)-3-Methylcyclohexanone	150	50	present	−6·1	+983	65/35
				—	+968	
(−)-3-Methylcyclohexanone	150	50	present	+7·0	−990	65/35
				—	−958	
(±)-3-Methylcyclohexanone	150	50	present	—	−600	65/35
				—	−580	
(±)-3-Methylcyclohexanone	150	16	trace	—	0	70/30
				—	0	
(±)-3-Methylcyclohexanone	150	25	present	—	—	65/35
4-Methylcyclohexanone	150	50	present	—	—	30/70

* These values were obtained from the ketones derived by chromic acid oxidation of the alcohol products, $[\alpha]_{305}^{MeOH}$ for the 2-position and $[\alpha]_{309}^{MeOH}$ for the 3-position.

† The unattacked ketone had $[\alpha]_{309}^{MeOH}$ +790.

and crystalline LADH (8 mg). The course of the reaction was determined at frequent intervals by spectrophotometric estimation of the amount of $NADH_2$ present. Reactions were allowed to proceed until all the coenzyme was consumed (10–15 h), the enzyme was then denatured by boiling for 5 min, after which the products were removed by continuous extraction with ether. The etheral extract was dried with $MgSO_4$. The components in the residue after removal of the solvent were separated by preparative gas chromatography, and purified by distillation at 30° under reduced pressure. Each fraction was identified by thin-layer chromatography, gas chromatography and optical rotation measurements. Alcohols were converted to ketones using the oxidizing agent of Curtis, Heilbron & others (1953) and their optical rotatory dispersion curves were traced. Where both the *cis*- and *trans*-isomers were produced the ratios were determined by measuring the areas under the respective curves of samples separated by analytical gas chromatography. In one experiment with (±)-3-methylcyclohexanone only 25 mg of the substrate was used and the reaction was terminated after 6 h. Table 3 lists the ketones studied, the amount of coenzyme used, the amount of unreacted ketone, the physical properties and identities of the products, and the ratio of *cis*- and *trans*-isomers formed.

Product formation from alcohols

The reactions were as described for the ketones except that in each case either 15, or 50 mg of the appropriate alcohol was oxidized with NAD (150 or 200 mg). The buffer used was 0·1M $Na_4P_2O_7$ at pH 9·8. Table 4 lists the compounds studied, the amount of enzyme used, the physical properties and identities of the products.

Metabolic experiments

These were as described by Elliott, Tao & Williams (1965) and the 24 h urine was hydrolysed by refluxing with 1N HCl for 1 h. The hydrolysate was then steam distilled, the distillate extracted with ether, dried with $MgSO_4$ and the ether removed.

Table 4. *Product formation from alcohol* in vitro

Compound	Amount of each reactant (mg) coenzyme	substrate	Unreacted alcohol	$[\alpha]^{MeOH}$*	Ketone formed
(±)-*cis*-2-Methylcyclohexanol	150	50	present	+260	(+) & (−)-2-,
	150	16	trace	0	(±)-2-,
(+)-*cis*-2-Methylcyclohexanol	150	50	present	−513	(−)-2-,
(±)-*trans*-2-Methylcyclohexanol	150	50	present	+150	(+) & (−)-2-,
	150	16	trace	0	(±)-2-,
(+)-*trans*-2-Methylcyclohexanol	150	50	present	+526	(+)-2-,
(±)-*cis*-3-Methylcyclohexanol	150	50	present	−470	(+) & (−)-3-,
	150	16	trace	−20	(+) & (−)-3-,
	200	16	undetected	0	(±)-3-,
(+)-*cis*-3-Methylcyclohexanol	150	50	present	−987	(−)-3-,
(−)-*cis*-3-Methylcyclohexanol	150	50	present	+960	(+)-3-,
(±)-*trans*-3-Methylcyclohexanol	150	50	present	−780	mainly (−)-3-,
	150	16	trace	−60	(+) & (−)-3-,
	200	16	undetected	0	(±)-3-,
(−)-*trans*-3-Methylcyclohexanol	150	50	present	+990	(+)-3-,
cis-4-Methylcyclohexanol	150	50	present	—	4-,
trans-4-Methylcyclohexanol	150	50	present	—	4-,

* 305 nm for the 2-ketone and 309 nm for the 3-ketone.

Table 5. *The metabolic products of the* 2-, 3-, *and* 4- *methylcyclohexanones and methylcyclohexanols* in vivo

Compound	Alcohol isolated	$[\alpha]_D$	$[\alpha]^{MeOH}$*	Ratio *cis*/*trans*
(±)-2-Methylcyclohexanone	(+)-*cis*-2	+8·9	−510	27/73
	(+)-*trans*-2	+22·4	+520	
(+)-2-Methylcyclohexanone	(+)-*trans*-2	+22·9	+512	0/100
(−)-2-Methylcyclohexanone	(+)-*cis*-2	+8·9	−508	100/0
(±)-3-Methylcyclohexanone	(±)-*cis*-3	0	0	70/30
	(±)-*trans*-3	0	0	
(+)-3-Methylcyclohexanone	((−)-*cis*-3	−5·9	+960	70/30
	(−)-*trans*-3	−8·2	+980	
4-Methylcyclohexanone	*cis*-4	—	—	30/70
	trans-4	—	—	
(±)-*cis*-2-Methylcyclohexanol	(+)-*cis*-2	+8·9	−524	20/80
	(+)-*trans*-2	+22·9	+520	
(±)-*trans*-2-Methylcyclohexanol	(±)-*trans*-2	0	0	0/100
(±)-*cis*-3-Methylcyclohexanol	(±)-*cis*-3	0	0	100/0
(±)-*trans*-3-Methylcyclohexanol	(±)-*cis*-3	0	0	70/30
	(±)-*trans*-3	0	0	
cis-4-Methylcyclohexanol	*cis*-4	—	—	30/70
	trans-4	—	—	
trans-4-Methylcyclohexanol	*trans*-4	—	—	0/100

* These values were obtained from the ketones derived by chromic acid oxidation of the alcoho products $[\alpha]_{305}^{MeOH}$ for 2-position and $[\alpha]_{309}^{MeOH}$ for the 3-position.

The residue was separated by preparative gas-liquid chromatography and the individual alcohols purified by distillation under reduced pressure. The characterization of the aglycones, and the determination of *cis*- and *trans*-ratios was as described for the products formed *in vitro* from ketones. A list of the compounds studied, the physical characterization of the aglycones, and the ratios of *cis*-alcohol to *trans*-alcohol formed is given in Table 5.

DISCUSSION

From an examination of Catalin molecular models representing possible coenzyme substrate interactions in the transition complex it can be seen that, of the theoretically infinite number of possible orientations for the substrate and coenzyme, if the enzyme is "A" stereospecific (Table 2) only six are effectively different. These are (i) the normal "face-to-face" orientation, in which the axial 3- and 5-hydrogen atoms of the substrate are directed towards the "A" face of the coenzyme, and C-1 of the substrate lies directly above C-4 of the coenzyme; (ii) the normal "head-to-tail" orientation in which the substrate, by rotation through 180° about a fixed point located at the carbonyl group lies in the same plane as the coenzyme, but with C-1 of the substrate and C-4 of the coenzyme facing each other; (iii) the normal "perpendicular" approach—an intermediate disposition between (i) and (ii)—arrived at by rotating the substrate molecule through 90° from the original face-to-face position. The remaining three dispositions are those in which the orientation of the substrate molecule relative to the coenzyme is reversed—that is the face bearing the 2,4,6-axial hydrogen atoms is presented to the coenzyme. To distinguish these from the normal orientations, they are termed "reverse face-to-face", "reverse head-to-tail" and "reverse perpendicular" orientations, and each substrate in reverse orientation subtends, relative to the coenzyme, exactly the same angle as the corresponding substrate in its normal orientation. Cheo & others (1967) have shown that the substrates react biologically

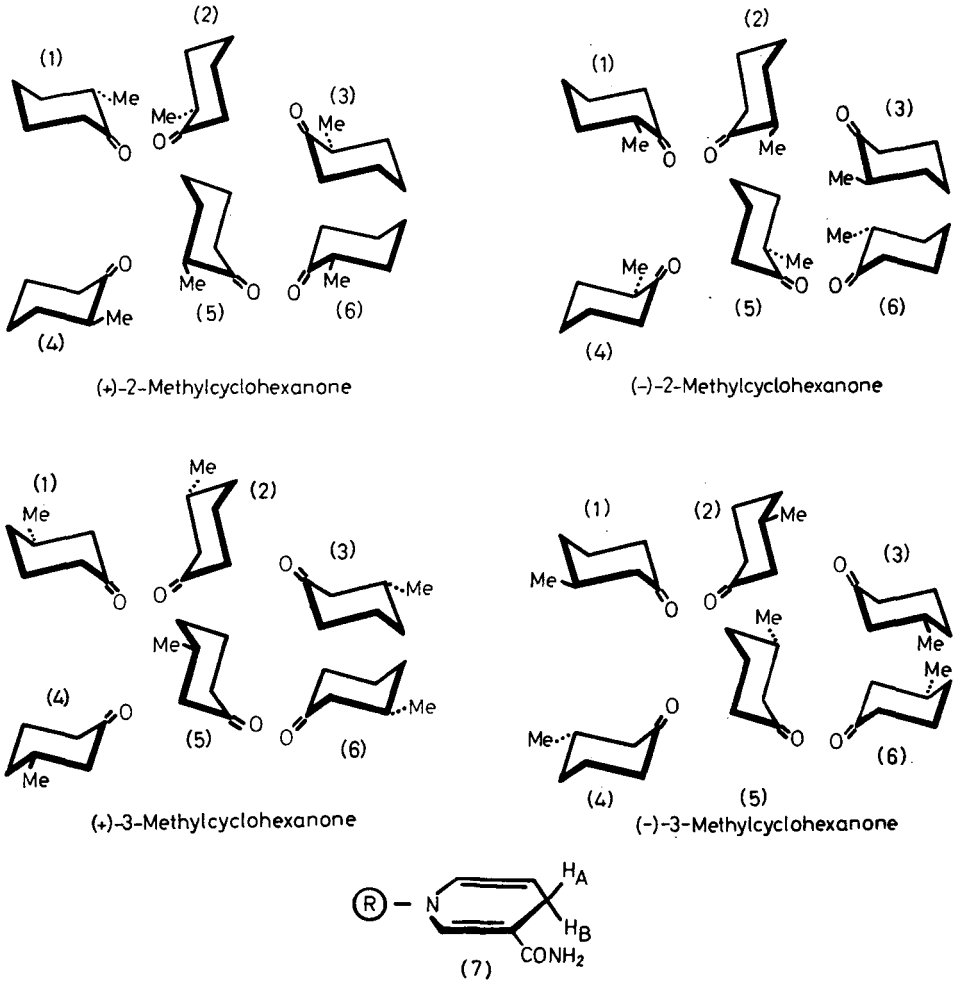

FIG. 1. Orientation of the 2-, and 3-methylcyclohexanones. (1) Face-to-face orientation, (2) Perpendicular orientation, (3) Head-to-tail orientation, (4) Reverse face-to-face orientation, (5) Reverse perpendicular orientation, (6) Reverse head-to-tail orientation. All orientations are relative to NADH (7) in the disposition shown.

in their most stable conformations and Fig. 1 demonstrates the various possibilities for the 2-, and 3-methylcyclohexanones. In these models it is considered that the function of the enzyme is to bind the coenzyme and substrate in the activated complex with the properties assigned to it by Vennesland (1958); the coenzyme is considered to be the asymmetric reagent.

Further examination of the models reveals that if there is to be a fruitful transfer of hydrogen, no steric interaction between the carboxyamide group of the nicotinamide moiety of the coenzyme and the alkyl substituent of the substrate should occur, and C-1 of the substrate should be accessible to the reactive "A" face of the coenzyme. Models indicate that, for ketones, the most important influence on hydrogen transfer is exercised by the steric interaction occurring between substituents on C-2 of the substrate and the carboxyamide group of the coenzyme, and it is this group that is decisive in determining the stereochemical course of the reaction. With alcohols,

because of their different configuration, carboxyamide interactions are demonstrably less significant, so that hydrogen transfers can occur that are impossible with ketones. However it is important to observe that if all experimentally found reaction products are to be accounted for correctly, the oxygen atom of the substrate must be directed away from the nitrogen atom of the nicotinamide ring as observed by McKinley-McKee (1964). Non-bonded hydrogen interactions are important only if they limit the accessibility of C-1. If these requirements are satisfied it is possible to predict not only that a reaction will occur, but also the identity of the product, if the stereochemical relation of the precursor to its product and the orientation of the substrate in the transition complex are known. In practice, the presence or absence of a carboxyamide interaction determines whether the (+) or (−)-stereoisomer, or both, will react, and the orientation in the substrate coenzyme complex determines whether a hydrogen atom is transferred to or from the "normal" or "reverse" side of the substrate. Transfer to the normal side of a ketone results in the formation of an equatorially orientated hydroxyl group, and transfer to the reverse side results in an axial hydroxyl group. Removal of hydrogen from an alcohol will result in the corresponding stereochemically related ketone.

Table 6 gives, for the 2- and 3-methylcyclohexanones and methylcyclohexanols, the only fruitful orientations of the various substrates that are without inhibitory interactions and in which the C-1 atom is accessible for hydrogen transfer. It also tabulates the predicted products in these orientations, the actual products obtained in enzymic experiments, and the experimental reversibility of each reaction. As shown in the Table, substrates react in only two of the six possible orientations—namely face-to-face

Table 6. *Orientations and interactions of the* 2 *and* 3*-methylcyclohexanones and methylcyclohexanols*

Substrate	Orientations without interaction	Predicted alcohol or ketone	Actual alcohol or ketone	Reversibility of reaction
(+)-2-Methyl-cyclohexanone	face-to-face only	(+)-*trans*-2-	(+)-*trans*-2-	complete
(−)-2-Methyl-cyclohexanone	reverse perp. only	(+)-*cis*-2-	(+)-*cis*-2-	complete
(+)-*trans*-2-Methylcyclo-hexanol	face-to-face only	(+)-2-methyl	(+)-2-methyl-	complete
(−)-*cis*-2-Methylcyclo-hexanol	reverse perp. only	(+)-2-methyl-	(+)-2-methyl-	irreversible
(−)-*trans*-2-Methyl hexanol	face-to-face only	(−)-2-methyl-	(−)-2-methyl-	irreversible
(+)-*cis*-2-Methyl-cyclohexanol	reverse perp.	(−)-2-methyl-	(−)-2-methyl	complete
(+)-3-Methyl cyclohexanone	face-to-face	(−)-*cis*-3-	(−)-*cis*-3-	complete
	reverse perp.	(−)-*trans*-3-	(−)-*trans*-3-	complete
(−)-3-Methyl-cyclohexanone	face-to-face	(+)-*cis*-3-	(+)-*cis*-3-	complete
	reverse perp.	(+)-*trans*-3-	(+)-*trans*-3-	complete
(−)-*cis*-3-Methyl-cyclohexanol	face-to-face	(+)-3-methyl-	(+)-3-methyl-	complete
(−)-*trans*-3-Methyl-cyclohexanol	reverse perp.	(+)-3-methyl-	(+)-3-methyl-	complete
(+)-*cis*-3-Methyl-cyclohexanol	face-to-face	(−)-3-methyl-	(−)-3-methyl-	complete
(+)-*trans*-3-Methyl cyclohexanol	reverse perp.	(−)-3-methyl-	(−)-3-methyl-	complete

and reverse perpendicular. For example with (+)-2-methylcyclohexanone the only orientation without interactions is face-to-face, in which orientation the predicted alcohol would be (+)-*trans*-2-methylcyclohexanol, and this is the alcohol that was isolated experimentally. Similarly (−)-2-methylcyclohexanone yields (+)-*cis*-2-methylcyclohexanol only, in a reverse perpendicular orientation as predicted. In the reverse reactions (+)-2-methylcyclohexanone is produced either as a result of a face-to-face reaction of (+)-*trans*-2-methylcyclohexanol, or by a reverse perpendicular reaction of (−)-*cis*-2-methylcyclohexanol, whilst (−)-2-methylcyclohexanone is produced as a result of either a face-to-face reaction of (−)-*trans*-2-methylcyclohexanol or a reverse perpendicular reaction of (+)-*cis*-2-methylcyclohexanol, and in each case the predicted and experimentally obtained products were the same. Where the reactions are completely reversible, [(+)-2-methylcyclohexanone ⇌ (+)-*trans*-2-methylcyclohexanol and (−)-2-methylcyclohexanone ⇌ (+)-*cis*-2-methylcyclohexanol] the substrates have the same orientation relative to the coenzyme in both the oxidized and reduced forms, whereas where the reactions are not reversible [(−)-*cis*-2-methylcyclohexanol → (+)-2-methylcyclohexanone and (−)-*trans*-2-methylcyclohexanol → (−)-2-methylcyclohexanone], an approach to the transition state from the ketone side is not possible because carboxyamide interactions prevent the ketone from participating. For a reaction to be reversible, the reacting species must have a common transition state. Further, since the two alcohols (−)-*cis*-2-, and (−)-*trans*-2-methylcyclohexanol produce ketones irreversibly, the geometry of the transition state, as Prelog has suggested (1964), must resemble that of the alcohol more closely than that of the ketone.

The most notable difference between the reactions of the 2-, and 3-methylcyclohexanones and methylcyclohexanols, is an apparent lack of stereospecificity in the reactions of the latter, due to the complete absence of carboxyamide interactions with all of these isomers in all possible orientations. Since the particular isomer produced is determined only by the accessibility of C-1, and the orientation of the substrate relative to the coenzyme, reactions with these ketones are possible in both normal and reverse orientations. It is predictable that both (+)- and (−)-3-methylcyclohexanone will react either in a face-to-face or a reverse perpendicular orientation, and in practice (+)-3-methylcyclohexanone yields (−)-*cis*-3-, and (−)-*trans*-3-methylcyclohexanol in a ratio of 65 : 35 whilst, (−)-3-methylcyclohexanone yields (+)-*cis*-3-, and (+)-*trans*-3-methylcyclohexanol also in a ratio of 65 : 35. Hence if the reaction with the racemic ketone (±)-3-methylcyclohexanone is allowed to go to completion, the two alcohols produced—*cis*-3-, and *trans*-3-methylcyclohexanol—will be racemic. If the reaction does not go to completion, as for example in a system where there is either excess of substrate, or a limited amount of coenzyme, the product will be partially optically active (Graves & others, 1965) and if the reaction proceeds for a short time, only one product may be isolated because the rates of the two reactions are not the same. The results in Table 3 demonstrate this point. Since the products obtained *in vivo* were invariably racemic, there must be adequate amounts of the coenzyme available to effect the biological transformation. Further, the finding that the *cis*-/*trans*-ratio (in the presence of excess of coenzyme) is 65 : 35 indicates that the face-to-face reaction occurs with greater ease than the reverse perpendicular reaction, the actual ratio observed being a direct comparative measure of the facility of the two reactions.

The present findings also support the earlier suggestion of Tao & Elliott (1962) that in the biological conversion of (±)-*cis*-2-methylcyclohexanol to the glucuronides of

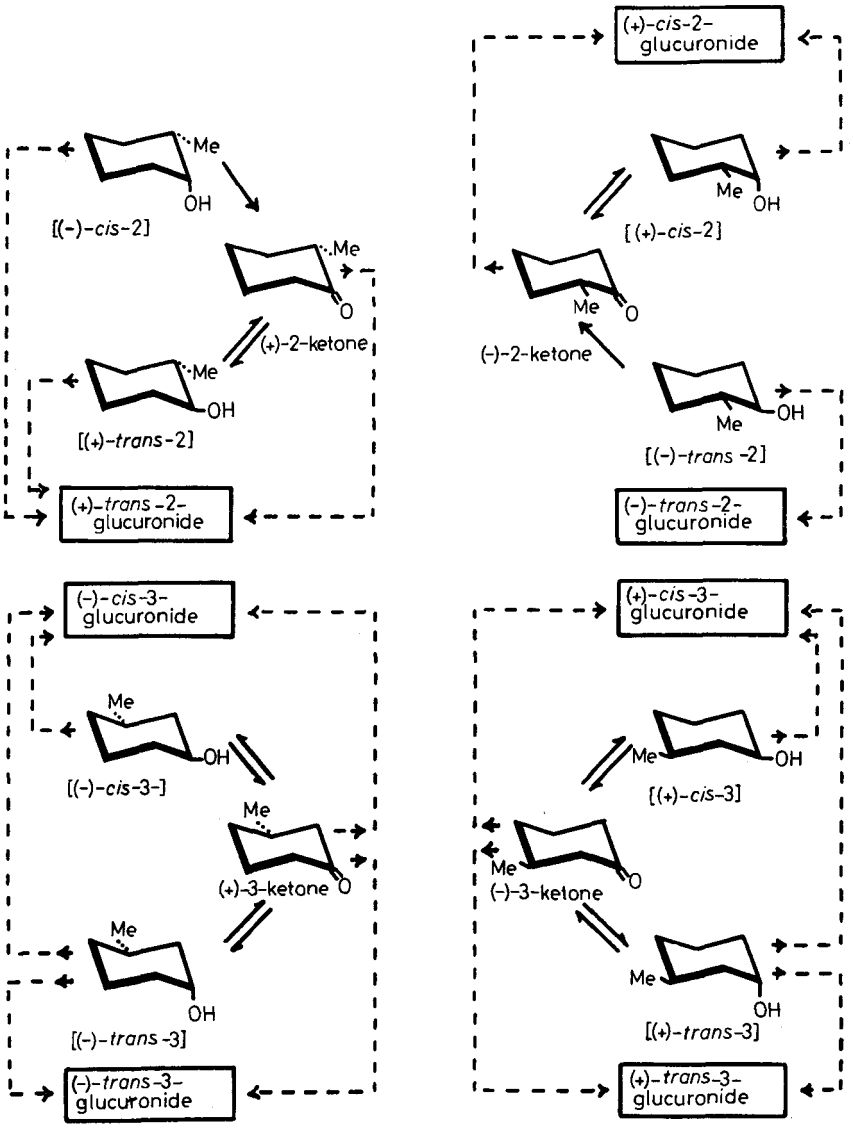

FIG. 2. *In vitro* (———) and *in vivo* (– – –) reactions of the 2- and 3-methylcyclohexanols and methylcyclohexanones.

(+)-*trans*-2- and (+)-*cis*-2-methylcyclohexanol and the similar conversions of (±)-*trans*-3-, and *cis*-4-methylcyclohexanols to the corresponding, thermodynamically more stable epimers, the inversions occur through a ketone intermediate. As shown in Table 5 when racemic *cis*-2-methylcyclohexanol is incubated with excess NAD and LADH the product is racemic 2-methylcyclohexanone and this, when incubated with liver alcohol dehydrogenase and excess $NADH_2$ (Table 3), is converted to (+)-*cis*-2-, and (+)-*trans*-2-methylcyclohexanol in a ratio of 28 : 72. Further confirmation is provided in the tritium transfer experiments in which NAD(T) was first reduced by (±)-*cis*-2-methylcyclohexanol to NADH(T) and in another experiment NADH(T) was used to reduce the ketone to the corresponding alcohols. Since radioactivity was

retained in the NADH(T) the hydrogen on C-1 of the (+)- and (−)-*cis*-2-methylcyclohexanols must have been successively transferred from the alcohols to the ketone and then to the (+)-*cis*- and (+)-*trans*-2-alcohols that were formed.

The *in vitro* results also afford an explanation of the particular metabolites found *in vivo*. Fig. 2 outlines the *in vitro* and *in vivo* reactions of the 2- and 3-methylcyclohexanols and methylcyclohexanones. The scheme indicates that whilst the (−)-*cis*-2-alcohol is rapidly converted *in vitro* to the (+)-2-ketone by an irreversible reaction (the Michaelis constant is particularly favourable) and thence by reduction to the (+)-*trans*-alcohol, finally to be conjugated with glucuronic acid, such a route is not possible for the (+)-*cis*-alcohol which can only be reversibly oxidized to (−)-2-methylcyclohexanone by a reaction having a relatively low affinity and low velocity in the reverse direction. Since the formation of the (−)-*trans*-alcohol from the (−)-ketone by an enzymic reaction has been shown not to occur; the only means of disposing of the (+)-*cis*-alcohol is by conjugation with glucuronic acid. Since the ratio of (+)-*cis*-2-, and (+)-*trans*-2-alcohols isolated from the urine was 27 : 73 it appears that the (+)-*cis*-2-alcohol accumulates in the body and is only slowly excreted. The intermediate conversion of the *trans*-2-alcohols to the 2-ketone *in vivo* is excluded because, had this occurred, the products would have been (+)-*trans*-2-, and (+)-*cis*-2-methylcyclohexanol (see Fig. 2). Not all of the enzymically possible reactions occur in the rabbit. For example the conversion of (−)-*trans*-2-methylcyclohexanol to (+)-*cis*-2-methylcyclohexanol, and the conversion of the *cis*-3-methylcyclohexanols, to the corresponding *trans*-3-methylcyclohexanols predictable on the basis of enzyme experiments, do not occur *in vivo*, the apparent reason being that the thermodynamically more stable alcohols are conjugated directly. The ketones, similarly, before conjugation are converted mainly into the more stable alcohol. These differences in the biological transformations indicate that the conjugation of equatorial alcohols with glucuronic acid occurs preferentially, and this is in accord with chemical conformational expectations.

The metabolic pattern of the 3-methylcyclohexanols is similar to that of the 2-methylcyclohexanols in that the (±)-*cis*-3-alcohols are conjugated in unchanged form, whilst the *trans*-3-alcohols are inverted presumably through a ketone intermediate, and the *in vitro* experiments duplicate the previously postulated pathway, viz. (±)-*trans*-3-methylcyclohexanol → (±)-3-methylcyclohexanone → (±)-*cis*-, and (±)-*trans*-3-methylcyclohexanol. Fig. 2 shows that all of the *in vitro* reactions unlike those of the 2-methylcyclohexanols, are completely reversible. Consequently, there is an equal possibility of all the alcohols being formed when racemic 3-methylcyclohexanone is fed, and this in fact occurs, (±)-*cis*-, and (±)-*trans*-3-methylcyclohexanols being formed in a ratio of 70 : 30.

Similar considerations apply in accounting for the inversions observed with the 4-methylcyclohexanols, and again the only assumption that needs to be made is that the inversions occur as a result of the formation of a ketone intermediate.

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