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STERIC MODE OF ENZYMIC HYDROXYLATION AT PRIMARY CARBON ATOMS[a]

HYDROXYLATION OF (1*R*)- AND (1*S*)-[1-^{3}H, ^{2}H, ^{1}H][1-^{14}C]-OCTANES BY *PSEUDOMONAS OLEOVORANS*†

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Abstract—(1*R*)- and (1*S*) [1-^{3}H, ^{2}H, ^{1}H]-octanes and mixed with [1-^{14}C]-octane, were synthesized. The mixed samples were incubated with homogenats of *P. oleovorans* strain TF4-1L and the biosynthesized mixtures of octanols isolated. It was shown that mainly the achiral termini [-C^{1}H$_3$] were hydroxylated and that chiral methyls were oxygenated to the extent of 20–30%. In all instances the products derived from hydroxylation at the chiral methyls [-C-^{3}H, ^{2}H, ^{1}H] were mixtures of (1*R*)- and (1*S*)-octanols, the major component of which was the alcohol obtained by displacement of ^{1}H. The results indicate that hydroxylation proceeded with a normal isotope effect $k_H > k_D > k_T$. The amount of (1*R*)-octanol in the mixtures of octanols derived from (1*R*)- and (1*S*)-octane was determined. It was found that the C-1 hydroxylation of octane proceeded with *retention*, i.e. the incoming hydroxyl assumed the orientation of the displaced hydrogen (or isotopic hydrogen) atom.

The pioneering concepts of Ogston[1] have greatly advanced out understanding of enzyme catalyzed reactions of *pro*-chiral molecules. For the current discussion we will assume that for a reaction to occur, a three-point attachment of substrate to the enzyme surface is required (Fig. 1). The particular group or *atom* of the substrate to undergo reaction must be bound at the *catalytic site* of the enzyme while two other groups are attached at their respective *binding sites*. The active site region of an enzyme constitutes a dissymmetric environment. Differences in the *size* and *shape* of substrate binding sites provide for the observed discrimination between enantiotopic groups of *pro*-chiral substrates. When binding a *pro*-chiral substrate $[C\text{-}R_1R_2X_aX_b][R_1 \neq R_2; X_a = X_b]$ an enzyme sterically distinguishes between R_1 and R_2 through its ability to accommodate them in the available binding sites. This imposes a particular steric orientation on the enzyme-substrate complex in which only one of the enantiotopic groups (in this case, X_a) is bound at the catalytic site (Fig. 1). Additionally, the reaction at the *catalytic site* may also be sterically controlled. The events at a pro-chiral center can be illustrated by the transformation of citrate to isocitrate by *cis*-aconitase which involves the *pro-R* hydrogen atom of the *pro-R* carboxymethyl branch of citrate.[2]

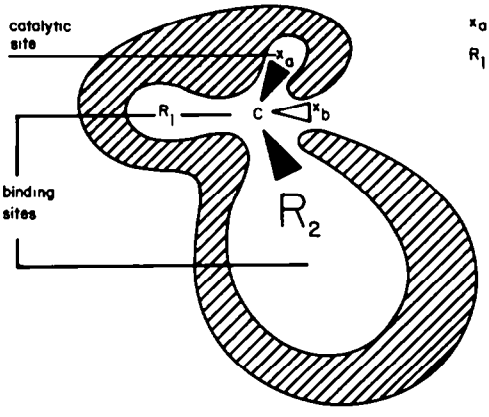

Fig. 1.

The above rationalization is not *sensu stricto* applicable to enzymic reactions at primary carbons of the type shown in Fig. 2 (H=D=T). A molecule possessing a freely-rotating methyl group has a statistically equal probability of binding to the enzyme with any of three hydrogen atoms in the catalytic site of the enzyme.

Because the steric differences between the isotopic hydrogen atoms of a chiral methyl group (Fig. 2) (D=^{2}H; T=^{3}H) are small, they will also have a statistically equal probability of binding at the catalytic site of the enzyme. Determination of the steric course of the enzymic reaction will be possible, however, if the transformation of the chiral methyl group involves a substantial kinetic isotope effect.

Our approach to the study of th esteric course of hydroxylation at primary carbon atoms was based

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[a]Dedicated to the memory of Robert Burns Woodward this paper will be included in the book version of the special Woodword Memorial Supplement.

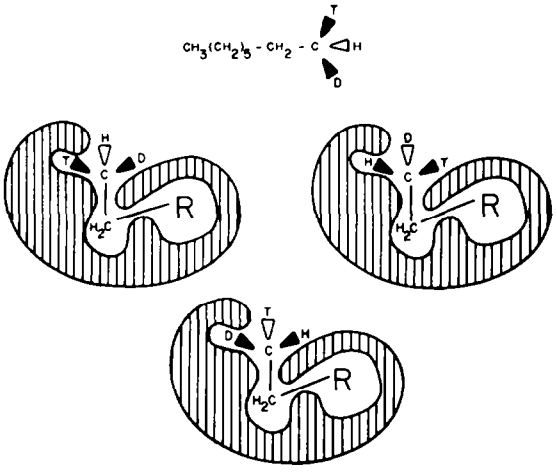


Fig. 2.

on the presumed operation of a normal kinetic hydrogen isotope effect $k_H > k_D > k_T$. We anticipated that enzymic hydroxylation at a chiral methyl group would proceed with such an isotope effect to yield alcohols of the type R–C–^{3}H, ^{2}H(OH) > R–C–^{3}H, ^{1}H(OH) > R–C–^{2}H, ^{1}H(OH). If the chirality of the methyl moiety of the substrate is known, then by determining the chirality of the predominant (tritated) alcohol, the steric course of the hydroxylation reaction could be deduced.

Since octane has been demonstrated to be efficiently metabolized to n-octanol by *Pseudomonas oleovorans* we selected (1*R*)- and (1*S*)-octanes as model substrates for the study of the stereochemistry of enzymic hydroxylation at a primary carbon atom.

Enzymic hydroxylation may proceed either with *retention*, *inverse*, or *racemization*. The terms "retention" and "inversion" are meant to indicate that the incoming hydroxyl assumes the same or the opposite orientation to the displaced hydrogen (or isotopic hydrogen) atom, respectively. The outcome of the hydroxylation of the (1*R*)- and (1*S*)-methyls of octane with retention and inversion, based on the operation of a normal kinetic hydrogen isotope effect, is summarized in Scheme 1. As the isotope effect involved in C–^{3}H bond breakage is usually large, little R–C–^{2}H, ^{1}H(OH) will be formed and can be disregarded. Besides, our analysis of the products of the reaction is based on the determination of the amount of (1*R*)[1–^{3}H]-alcohol present in the mixture (see below), and this precludes the detection of any species not tritiated at C-1.

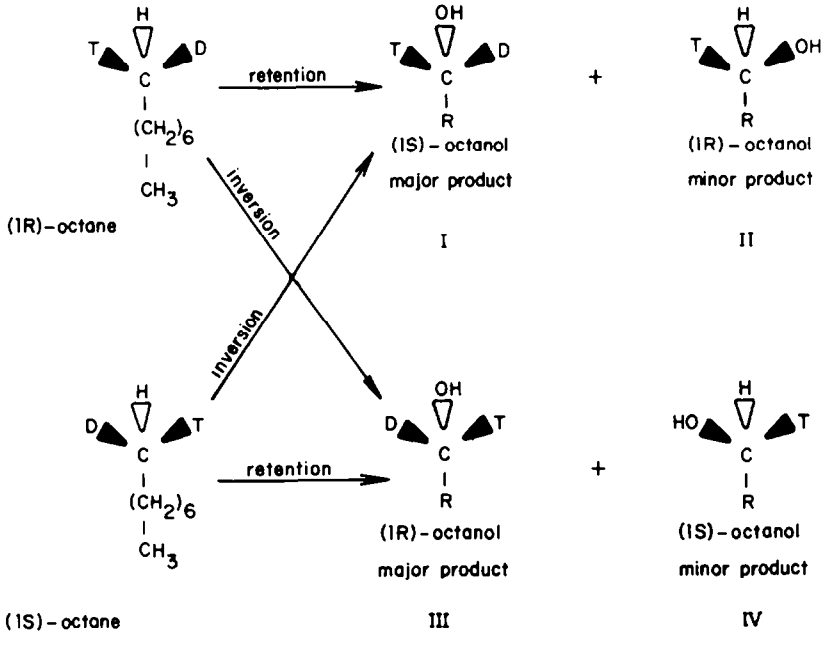
If hydroxylation proceeds with *retention* and $k_H > k_D$, then the major product derived from (1*R*)-octane will be (1*S*)-octanol (**I**), with a lesser amount of (1*R*)-octanol (**II**). Were the reaction to proceed with *inversion*, however, the major product would be (1*R*)-octanol (**III**) and the minor product (1*S*)-octanol (**IV**) (Scheme 1).

By the same reasoning, hydroxylation of (1*S*)-octane with *retention* would yield (1*R*)-octanol (**III**) as the major product and (1*S*)-octanol (**IV**) as the minor product, while for *inversion* the major and minor products would be (1*S*)-octanol (**I**) and (1*R*)-octanol (**II**), respectively.

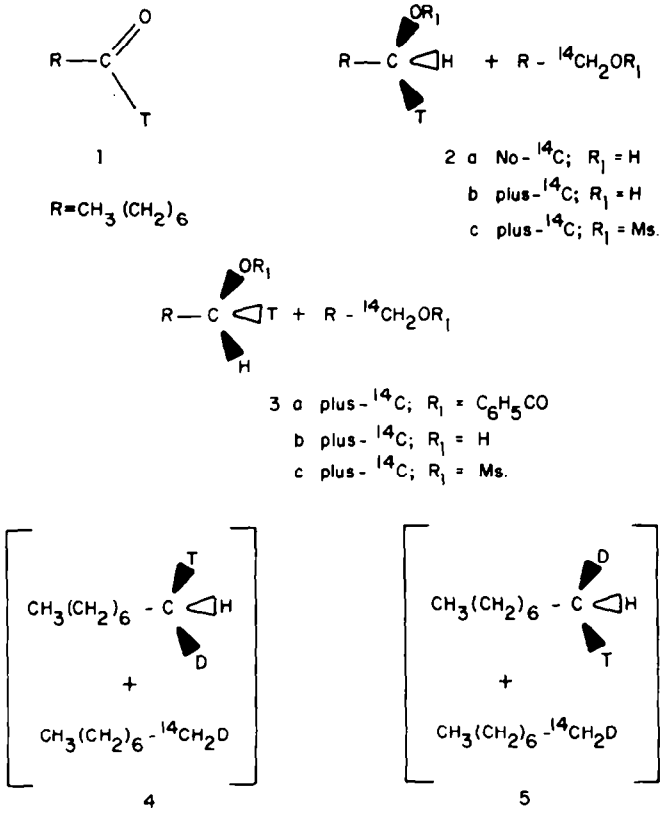
Should hydroxylation proceed with racemization, or stereospecifically in the absence of an isotope effect, then equal amounts of (1*R*)- and (1*S*)-octanols would be formed, *regardless of the chirality of the substrate octane.*

A solution to the problem of the stereochemistry of octane hydroxylation required: (i) (1*R*)- and (1*S*)-octanes; (ii) a method for enzymic production of 1-octanols from chiral octanes; (iii) a procedure for analyzing the chiral composition of octanols derived from oxygenation at the *chiral* methyls.

We first addressed the problem of synthesis of (1*R*)- and (1*S*)-octanes. Our approach was to syn-



Scheme 1.



Flow Sheet 1.

thesize (1*S*)- and (1*R*) [1-^{3}H, ^{1}H]-octanols,[3] convert them to the corresponding mesyl esters, and finally hydrogenolyze the (1*S*)- and (1*R*)-mesyl esters with lithium triethylborodeuteride ($LiBEt_3D$) to (1*R*)- and (1*S*) [1-^{3}H, ^{2}H, ^{1}H]-octanes, respectively. The methodology for the syntheses of the key intermediates, (1*S*)- and (1*R*)-octanols, was developed with deuterated compounds and was reported in an earlier paper.[3] For the current study two modifications were introduced.

To avoid the troublesome hydrolysis of a [1-^{3}H]-octanal thioacetal,[3] the aldehyde (**1**) (Flow Sheet 1) was prepared by oxidation of (1*RS*)-(1-^{3}H]-octanol. The second modification involved the use of sodium dithionite to recycle NAD to NADH in the horse liver alcohol dehydrogenase (HLAD) reduction[4] of [1-^{3}H]-octanal (**1**). The aldehyde was reduced in batches of *ca.* 1 g to give (1*S*) [1-^{3}H]-octanol containing no more than 10% of [1-^{3}H]-octanal.

The alcohol was freed of aldehyde (**1**) by column chromatography to yield (1*S*) [1-^{3}H]-octanol (**2a**) (*ca.* 50% yield from (1*RS*)-octanol). To the homogeneous (1*S*)-octanol (**2a**), [1-^{14}C]-octanol was added and the [^{3}H: ^{14}C]-ratio of the mixed sample (**2b**) was determined (as the 3,5-dinitrobenzoate ester). Jones' oxidation of (1*S*) [1-^{3}H][1-^{14}C]-alcohol to octanoic acid proceeded with the loss of more than 99% of tritium, indicating that octanol, and hence the derived octanes, contain less than 1% of tritium at sites other than C-1.

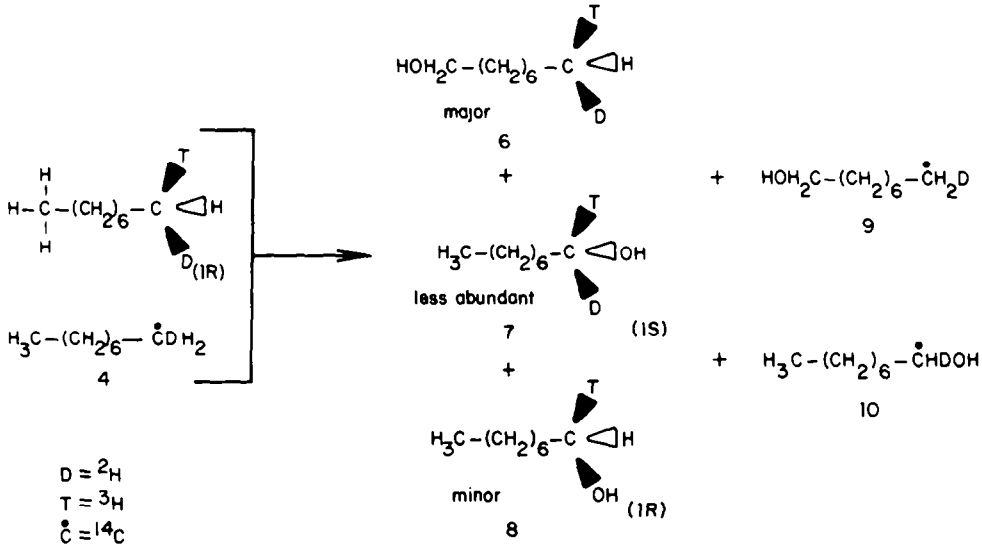
Treatment of (1*S*) [1-^{3}H][1-^{14}C]-octanol (**2b**) with a mixture of diethyl azodicarboxylate, benzoic acid and triphenylphosphine in THF gave (1*R*) [1-^{3}H][1-^{14}C]-octyl benzoate[3] (**3a**) (90% yield). The (1*R*)-ester (**3a**) was reduced with $LiAlH_4$ to give, after column chromatography, (1*R*)-octanol (**3b**) (50% yield from **2b**).

The enantiomeric purity of the (1*R*) [1-^{3}H][1-^{14}C]-octanol (**3b**) was assayed by oxidation with HLAD-NAD to octanal. Oxidation to octanal proceeded with the loss of 95–96% of tritium, indicating that the alcohol (**3b**) contained at least 95–96% of (1*R*) [1-^{3}H]-octanol. By implication, the parent (1*S*) [1-^{3}H][1-^{14}C]-octanol (**2b**) must have contained at least 95–96% of the (1*S*)-alcohol. These results are in full accord with the results of our model studies with (1*R*)- and (1*S*) [1-^{2}H]-octanols, the chiral purities of which were also determined by (^{1}H)-NMR.[3]

At this point we noticed that the alcohol dehydrogenase-NAD oxidation procedure could not be used for the determination of the enantiomeric purity of (1*S*) [1-^{3}H][1-^{14}C]-octanol (**2b**). We observed that, irrespective of whether horse liver, yeast, or *P. oleovorans* alcohol dehydrogenase was used, the [^{3}H: ^{14}C]-ratios of the resulting aldehydes were higher (up to 40%) than the [^{3}H: ^{14}C]-ratio of the (1*S*) [1-^{3}H][1-^{14}C]-octanol (**2b**) substrate. It is likely that the observed increase in [^{3}H: ^{14}C]-ratio is caused by further oxidation of the obtained octanals to octanoic acid.[5] Possibly the produced [1-^{3}H]-octanal (R-C^{3}HO) is oxidized to octanoic acid at a considerably *slower rate* than [1-^{14}C]-octanal (R-^{14}C^{1}HO); hence, at any point in time (ranging from 7 min to 24 h), the

mixture is depleted of [^{14}C]-aldehyde, and the isolated octanal shows a higher [^{3}H: ^{14}C]-ratio than the starting alcohol (**2b**).

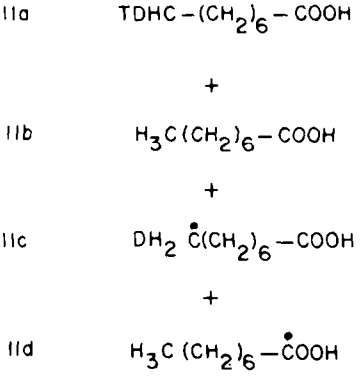
The samples of (1*S*) [1-^{3}H][1-^{14}C]-octanol (**2b**) and (1*R*) [1-^{3}H][1-^{14}C]-octanol (**3b**) were converted to the corresponding mesyl esters [(**2c**) and (**3c**)], treatment of which with $LiBEt_3D$ in diglyme gave (1*R*) [1-^{3}H, ^{2}H, ^{1}H][1-^{14}C]-octane (**4**) and (1*S*) [1-^{3}H, ^{2}H, ^{1}H][1-^{14}C]-octane (**5**), respectively.[6]



Flow Sheet 2.

Having secured the required chiral substrates, we focused our attention on enzymic hydroxylation. Let us consider the composition of the radioactive alcohols obtained if the enzymic hydroxylation of (1*R*)[1-^{3}H, ^{2}H, ^{1}H][1 - ^{14}C]-octane (**4**) proceeds with retention (Flow Sheet 2). If $k_H > k_D > k_T$, then the major product will be (**6**) resulting from oxygenation at the achiral methyl terminus (-C^{1}H$_3$). The main product of attack at the *chiral* methyl will be alcohol (**7**) arising from displacement of the C-1 hydrogen atom. In addition, a smaller amount of alcohol (**8**), obtained by displacement of a deuterium atom, will be formed. The [^{14}C]-octanols (**9**) and (**10**) are internal standards against which the loss of tritium is measured. As explained above, we will disregard the small amount of product obtained by C-^{3}H bond breakage.

The stereochemistry of the hydroxylation can only be related to tritiated products of hydroxylation at the chiral methyl, namely octanols (**7**) and (**8**). For determination of the extent of oxygenation at the chiral terminus, the mixture of biosynthesized alcohols (**6–10**) was oxidized with Jones' reagent to give a mixture of acids (**11a–d**). Since only C-1 chiral octanols (**7**) and (**8**) lost tritium in this oxidation, differences between the [^{3}H: ^{14}C]-ratio of the mixture of alcohols (**6–10**) and the mixture of acids (**11a–d**) gave the extent of oxidation at the chiral terminus of octane. This also defined the maximal amount of C-1 tritium potentially available for enzymic exchange in the chirality determination.



Flow Sheet 3.

As indicated above, alcohol dehydrogenase-NAD could not be used for assaying the chirality of a mixture of biosynthesized octanols. We therefore examined the equilibration procedure of primary alcohols catalyzed by HLAD-NAD and porcine heart diaphorase.[7] This method is based on the premise of exclusive exchange of the 1-*pro*-R hydrogen atom of alcohol with water.[8] In our hands, both (1*R*)- and (1*S*)-tritium atoms of [1-^{3}H]-octanols were exchanged, although the (1*S*)-tritium atom was exchanged at a considerably slower rate. Under the experimental conditions employed by us, (1*R*) [1-^{3}H, ^{1}H][1-^{14}C]-octanol lost essentially all its tritium in 12 h. To assure the *complete* exchange of the (1*R*) tritium the reaction was allowed to proceed for 24 h, during which time (1*S*) [1-^{3}H, ^{1}H][1-^{14}C]-octanol (**2b**) lost 30–40% of its tritium and (1*RS*) [1-^{3}H, ^{1}H][1-^{14}C]-octanol lost 65–70% of tritium. Since (1*RS*)-octanol contains only 50% of (1*S*) [1-^{3}H, ^{1}H]-octanol, it follows that 30–40% of the tritium of the (1*S*)-enantiomer was lost after 24 h. In parallel control experiments with octanols derived from (1*R*)- and (1*S*)-octanes and synthetic (1*RS*)-octanol, the loss of C-1 tritium was reproducible within a narrow range (Table 1). Hence, by sub-

Table 1. Determination of [^{3}H: ^{14}C] ratios of octanols of different origin following incubation with HLAD, NAD, NADH and diaphorase for 24 hr (see text)

	SOURCE OF OCTANOL		
	HYDROXYLATION OF OCTANE		SYNTHETIC
SAMPLE	(1R)	(1S)	(1RS)
	$^3H:^{14}C$	$^3H:^{14}C$	$^3H:^{14}C$
1	7.33	6.65	1.84
2	7.51	6.55	2.09
3	7.47	6.49	2.01
Av.	7.44	6.56	1.98

tracting the amount of tritium lost from (1*S*)-octanol from the total amount of tritium lost during equilibration, the fraction of (1*R*)-octanol in the biosynthetic mixture can be computed.

We indicated above that the exchange procedure was purposely allowed to proceed for 24 h to *assure the complete* exchange of the tritium of (1*R*)-octanol. Since tritium has the *largest* kinetic hydrogen isotope effect, then by allowing the exchange of tritium from (1*R*)-octanol to proceed to completion secondary isotope effects of C-1 deuterium atoms are overridden. To test this hypothesis, we equilibrated in parallel under identical conditions (1*RS*) [1-^{3}H, ^{1}H][1-^{14}C]-octanol and (1*RS*) [1-^{3}H, ^{2}H][1-^{14}C]-octanol (99% 1-$^2H_1$). In both instances, identical amounts of tritium (64% and 65%, respectively) were lost in 24 h. This validates the outlined method of determining the amount of (1*R*)-octanols in the biosynthesized mixtures containing R-C-^{3}H, ^{2}H(OH) and R-C-^{3}H, ^{1}H(OH)-octanols of opposite configuration. It should be stressed, however, that conclusions on the course of the reaction could only be drawn from parallel hydroxylation experiments of (1*R*)- and (1*S*)-octanes. To be valid, the results of the two sets of experiments must be complementary.

For determination of the chirality of products of enzymic hydroxylation, the mixture of the octanols obtained from (1*R*) [1-^{3}H, ^{2}H, ^{1}H][1-^{14}C]-octane (4) and (1*S*) [1-^{3}H, ^{2}H, ^{1}H][1-^{14}C]-octane (5), and (1*RS*) [1-^{3}H] [1-^{14}C]-octanol were equilibrated in parallel under identical conditions. The equilibrated alcohols were recovered and converted to 3,5-dinitrobenzoate esters, which were extensively purified by tlc and multiple crystallizations for the [^{3}H: ^{14}C]-ratio determinations. The correction for the fraction of tritium lost from biosynthetic (1*S*)-alcohols was calculated on the basis of tritium lost from (1*RS*) [1-^{3}H, ^{1}H][1-^{14}C]-octanol. The results are summarized in Table 2.

Oxidation of the biosynthesized octanols derived from (1*R*)- (4) and (1*S*)-octane (5) with Jones' reagent gave octanoic acids and involved the loss of 20–30% of the tritium (Column 2, Table 2). This revealed that oxygenation of the octanes occurred mainly at the achiral methyl (-C$^1H_3$) to give, e.g. (6). The results are in accord with the anticipated operation of a normal kinetic hydrogen isotope effect.

The average deviation in the [^{3}H: ^{14}C] ratios from successive crystallization of the octanol and octanoic acid derivatives [see Table 4, Experimental] is ± 0.05. The resulting *relative* error[9] in the calculated percent of (1*R*)-octanol (Column 4, Table 2) is ± 7%.

Equilibration of the mixture of octanols derived from (1*R*)-octane (4) proceeded with the net (corrected) loss of 37% ± 3% and 38% ± 3% of the tritium available at C-1 (Table 2). This establishes that the main product of oxygenation of the (1*R*)-methyl of octane (4) is (1*S*)-octanol (7) and the minor product is (1*R*)-octanol (8). These results are in full agreement with the hypothesis that the reaction proceeds with retention and $k_H > k_D$.

The equilibration of products of oxygenation of (1*S*)-octane (5) occurred with the net (corrected) loss of 74% ± 5% and 82% ± 6% of the tritium available at C-1. It follows that the main product of oxygenation at the (1*S*)-methyl of octane (5) is (1*R*)-octanol (Scheme 1; III), with (1*S*)-octanol (Scheme 1; IV) the minor product. Again, the results are in agreement with the view that hydroxylation proceeds with retention and $k_H > k_D$.

The above results show that attack by hydroxy-

Table 2. Hydroxylation of (1*R*) and (1*S*) [1-^{3}H, ^{2}H, ^{1}H][1-^{14}C]-octanes by homogenates of *Pseudomonas oleovorans* strain TF4-1L

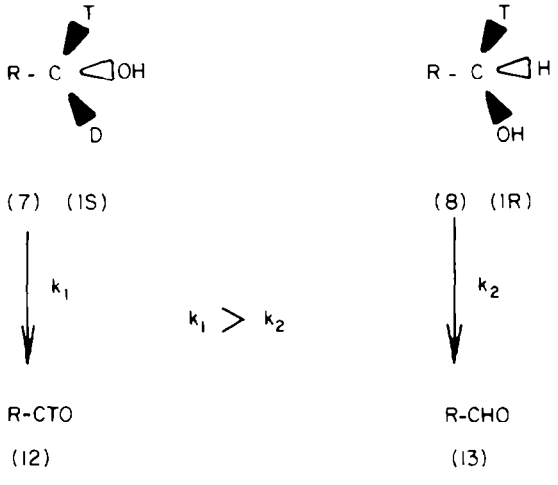
EXPERIMENT	CHIRALITY OF OCTANE	% OF -^{3}H AT C-1 OF OCTANOLS	% of (1R)-OCTANOL*	C-1 CHIRALITY OF THE MAJOR OCTANOL	STEREOCHEMISTRY OF HYDROXYLATION
1	1R	26	38 ± 3	1S	Retention
	1S	22	82 ± 6	1R	Retention
2	1R	29	37 ± 3	1S	Retention
	1S	29	74 ± 5	1R	Retention

* The (±) limits indicate the estimated error calculated on the basis of the variations of [^{3}H: ^{14}C]-ratios during several crystallizations of the derivatives listed in Table 4.

lase proceeded predominantly at the achiral methyls of (4) and (5). The oxygenation of an (*R*)- or (*S*)-methyl invariably produced (complementary) chiral mixtures of C-1 alcohols, the major component of which was obtained by *removal of* ^1H. These results are consistent with the operation of a kinetic isotope effect $k_H > k_D > k_T$ and, indicate that the hydroxylation proceeds with retention.

We considered the possibility that the endogenous alcohol dehydrogenase in the *P. oleovorans* cell-free extract could selectively utilize one of the biosynthesized octanol isomers. This might distort the isomeric composition of the recovered octanols and so lead to erroneous conclusions. Although we expected the dehydrogenase to utilize primarily the large amount of C-1 protiated alcohols derived from the oxidation of [1-^{14}C]-octane and from the archiral methyl of chiral octane, a selective attack on the C-1 chiral alcohols could still not be excluded *a priori*.

To clarify this point we will again consider the pool of products (**6–10**) obtained by oxygenation of (1*R*) [1-^{3}H, ^{2}H, ^{1}H][1-^{14}C]-octane. Octanols (**6**), (**9**), and (**10**) can be disregarded since their oxidation by the dehydrogenase has no bearing on the overall C-1 chirality of the pool of octanols. The oxidation of (1*S*) [1-^{3}H, ^{2}H][1-^{14}C]-octanol (**7**) by *P. oleovarans* alcohol dehydrogenase to octanal (**12**) will involve abstraction of the (1*R*)-deuterium atom while oxidation of the (1*R*) [1-^{3}H, ^{1}H][1-^{14}C]-octanol (**8**) to (**13**) will proceed with the loss of a tritium atom (Scheme 2). It is likely, therefore, that the rate of oxidation (k_1) of (**7**) will be greater than the rate of oxidation (k_2) of (**8**) ($k_1 > k_2$). It follows that at any point in time a faster depletion of the (1*S*)-octanol (**7**) could occur. However, our results distinctly and unambiguously show that the (1*S*)-octanol (**7**) is the main product of oxidation of (1*R*)-octane and that (1*R*)-octanol (**8**) is the minor product. This is in full agreement with the view that the oxygenation of (1*R*)-octane proceeds with retention. It also proves that (1*S*)-octanol was not removed from the pool of biosynthesized octanol in sufficient amounts to obscure the results. The same rationalization is equally applicable to the pool of octanols obtained from (1*S*)-octane.



Scheme 2.

EXPERIMENTAL

Materials. Hores liver alcohol dehydrogenase (EC 1.1.1.1, lot #128C-8050, 1.9 U/mg protein), ovalbumin (grade V), NAD (grade III), NADH (grade III), and DL-α-lipoamide were purchased from Sigma Chemical Co. (St. Louis, MO). Porcine heart diaphorase (EC 1.6.4.3, grade I, control No. 1319322/Sep. 1980) was obtained from Boehringer Mannheim GmbH (Mannheim, West Germany). ^3H_2O (5 Ci/mL) and $Ba^{14}CO_3$ (25 mCi, 55 mCi/mmol) were obtained from International Chemical and Nuclear Corp. (ICN) (Irvine, CA). Lithium triethylborodeuteride (Suber Deuteride, 1M solution in THF) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Silica gel 60 HF 254 + 366 and silica gel 60 (70–230 mesh) (both purchased from E. Merck A. G., Darmstadt, West Germany), were used for thin-layer chromatography and column chromatography, respectively.

Liquid scintillation counting was performed in a Mark II Liquid Scintillation System (Nuclear-Chicago, Des Plaines, IL), using 15 mL of Liquifluor (New England Nuclear, Boston, MA). Thin-layer radiochromatograms were scanned with an Actigraph III (Nuclear-Chicago) or Packard model 7200 radiochromatograph. Analytical gc was performed using a model 3700 gas chromatograph (Varian Associates, Inc., Walnut Creek, CA) fitted with a Varian stainless steel 3% OV-17 Chrom W-HP column (80–100 mesh, 2 m length × 2 mm width).

(1RS) [1-^{3}*H*]-*Octanol.* To $LiBH_4$ (13.4 mmol) in 27.9 mL THF was slowly added 200 μL of ^3H_2O (11.1 mmol, 1 Ci in 5 mL of THF) and the solution was refluxed for 1 h. To the cooled mixture octanal (4.4 g, 34.3 mmol) in 10 mL of diethyl ether was added dropwise. The reaction mixture was stirred for 30 min at room temperature and concentrated *in vacuo*. 1N HCl (13.4 mL) was added to dissolve the inorganic residue and the mixture was briefly warmed. The mixture was neutralized with 1N NaOH (13.4 mL) and extracted with pentane (3 × 30 mL). The pentane was dried (Na_2SO_4) and concentrated *in vacuo* to a colorless liquid; yield of (1*RS*) [1-^{3}H]-octanol (4.04 g; 238 mCi of ^{3}H).

[1-^{3}*H*]-*Octanal* (**1**). To a suspension of 8 g (21 mmole) of pyridinium dichromate[11] in 50 mL of dry CH_2Cl_2 was added 2.3 mL (15 mmole, 79 mCi) of (1*RS*) [1-^{3}H]-octanol. The mixture was stirred at room temp. until gc of an aliquot showed the absence of alcohol (12 h), then dry ether was added (100 mL). The mixture was filtered and the brownish filtrate stored overnight at −20°C. The solution was concentrated under reduced pressure to a small volume and filtered through a column of silica gel (8 g) which was then rinsed sequentially with pentane (50 mL) and ether (50 mL). The combined eluate was concentrated *in vacuo* (below room temp.) to give the aldehyde (**1**) as a colorless oil.

(1S) [1-^{3}*H*]-*Octanol* (**2a**). A solution of HLAD (40 mg) and NAD (160 mg) in 0.2 M potassium phosphate (pH 6.6; 750 mL) was rapidly stirred and flushed with N_2 (30 min.) in a round bottom flask (2L). A solution of sodium dithionite (15 g) in water (125 mL) in a pressure equalization funnel was flushed with N_2 (15 min). The sodium dithionite solution was added dropwise to the enzyme solution while flushing with N_2 continued. Initially the mixture developed a greenish-yellow tinge, which disappeared after a few minutes. The flow of N_2 was discontinued and about half of the synthesized aldehyde was injected into the sealed reactor, followed by injection of the ether washing (0.5 mL) of the syringe. After stirring the mixture at ambient temperature (8 h), a second portion of sodium dithionite (15 g in 100 mL H_2O; flushed with N_2) was added dropwise. The reaction was terminated after *ca.* 27 h, NaCl (100 g) was added and the product recovered with CH_2Cl_2 (5 × 75 mL). The combined CH_2Cl_2 extract was dried (Na_2SO_4) and con-

centrated (*ca.* 10 mL). GC indicated less than 10% aldehyde (**1**) remaining. Two incubation experiments were carried out and the extracts combined. The combined extract was fractionated on a silica gel column (100 g) prepared with pentane. The aldehyde (**1**) was eluted with pentane–ether (99:1) and (1*S*) [1-^{3}H]-octanoll (**2a**) (40 mCi) was eluted with pentane-ether (9:1).

[1-^{14}C]-Octanol was added to (1*S*) [1-^{3}H]-octanol (**2a**) (*ca.* 35 mCi of ^{3}H) and an aliquot of the resulting (1*S*) [1-^{3}H][1-^{14}C]-octanol (**2b**) was converted to octyl-3,5-dinitrobenzoate. The ester showed a [^{3}H: ^{14}C]-ratio of 9.27. The stock (**2b**) was used in subsequent experiments.

(1R) [1-^{3}*H*][1-^{14}*C*]-*Octanol* (**3b**). A mixture of (1*S*) [1-^{3}H][1-^{14}C]-octanol (**2b**) (20 mCi of ^{3}H; *ca.* 2.7 mmole), benzoic acid (500 mg; 4.2 mmole), and diethyl azodicarboxylate (990 μL; 5.2 mmole) in dry THF (5 mL) was stirred, cooled in an ice bath and flushed with N_2. To the cooled mixture, a solution of triphenyl-phosphine (1.64 g; 6.3 mmole) in THF (5 mL) was added dropwise and the reaction stirred at ambient temperature (16 h) in an atmosphere of N_2. Most of the volatile components were removed under reduced pressure and the resulting semisolid residue was leached with pentane (6 × 5 mL). The combined pentane extract was filtered and concentrated. The obtained product was fractionated on a column of silica gel (35 g) which was percolated with pentane (1L) until significant amounts of ^{3}H were detected in the eluate. The solvent was then changed to pentane–ether (99:1) and (1*R*) [1-^{3}H][1-^{14}C]-octyl benzoate (**3a**) (18 mCi of ^{3}H) was eluted (700 mL).

The eluates containing **3a** were concentrated (*ca.* 2 mL) and added to a solution of $LiAlH_4$ (111 mg; 2.9 mmole) in ether (6 mL). The mixture was refluxed (3 h) then more $LiAlH_4$ (40 mg) was added and refluxing continued (1.5 h). The reaction was terminated with water, acidified (6N HCl) and the products recovered with ether (3 × 3 mL). The extract was processed in the conventional manner and concentrated (*ca.* 8 mL). The obtained solution was applied to a column of silica gel (125 g) prepared with pentane. The column was eluted sequentially with pentane–ether (99:1) (5.5 L); pentane–ether (97.5:2.5) (8 L) and pentane–ether (97:3) (2 L) at which time the octanol appeared in the eluate. The octanol was then eluted with the same solvent mixture (4 L) after which benzyl alcohol began to co-elute with the octanol. The eluates containing the (1*R*) [1-^{3}H][1-^{14}C]-octanol (**3b**) were combined and concentrated (*ca.* 5 mL, 11 mCi of ^{3}H).

An aliquot of (**3b**) was converted to octyl 3,5-dinitrobenzoate, which showed a [^{3}H: ^{14}C]-ratio of 8.81.

(1R)-(4) *and* (1S)-5-[1-^{3}*H*, ^{2}*H*, ^{1}*H*][1-^{14}*C*]-*Octanes*. A 1 M solution of lithium triethylborodeuteride in THF (10 mL) was concentrated under reduced pressure and dry diglyme (9 mL) added under argon. A mixture of the appropriate chiral octanol [(**2a**) or (**3a**)] [*ca.* 1.1 mmole; 7–8 mCi of ^{3}H], triethylamine (450 μL; 3.2 mmole) in dry CH_2Cl_2 (5 mL) was cooled (ice-acetone) under N_2, and a solution of methanesulfonyl chloride (225 μL; 2.9 mmole) in CH_2Cl_2 (1 mL) was added dropwise (10 min.). The reaction was stirred at −20° for 15 min, by which time all the alcohol was consumed, as evidenced by tlc (silica gel; CH_2Cl_2). The plate was scanned for radioactivity and no radioactivity was detected in the octanol zone (R_f 0.15) (R_f of octyl mesylate 0.54).

The mixture was diluted with ether (10 mL), washed with ice cold H_2O (5 mL); 1N HCl; 5% aq. $NaHCO_3$ (2 × 5 mL); brine (5 mL), then dried and concentrated (*ca.* 1 mL).

Ether (5 mL) was added to the mesylate solution [(**2c**) or (**3c**)], followed by lithium triethylborodeuteride in diglyme (8 mL). The mixture was stirred at room temperature (1 h), at which time a trace of mesylate was still detectable (tlc). More lithium triethylborodeuteride in diglyme (1 mL) was added and stirring was continued (30 min) until the mesylate was completely reduced (tlc).

The mixture was cooled in ice and 2.5 N NaOH (4 mL) was added slowly (exothermic reaction), followed by 30% H_2O_2 (3.7 mL) (very exothermic). Ether (5 mL) was added and the mixture distilled through a Vigreux column (15 cm). The ether and residual CH_2Cl_2 were distilled at 35°, then the temperature of the bath was increased and 10 mL of distillate was collected at 75°–100°C. The distillate was saturated with NaCl, transferred to a 25 mL volumetric flask and saturated NaCl added to bring the upper (organic) layer (*ca.* 1 mL) into the neck of the flask. The organic layer was withdrawn and transferred to a 5 mL volumetric flask containing ice cold concentrated H_2SO_4 (2.5 mL). Additional cold concentrated H_2SO_4 was added to bring the octane into the neck of the flask. The sulfuric acid procedure was repeated several times and the obtained octane (95–100 mg; 7.3 mCi per mmole of ^{3}H) was stored in a vial over a few crystals of K_2CO_3.

Determination of the [1-^{3}*H*]-*Octanol Content in* (**2b**). Octanol (250 mg) was added to a solution of (1*S*) [1-^{3}H][1-^{14}C]-octanol (**2b**) (10 μCi of ^{3}H) in acetone (1 mL). The mixture was cooled in an ice bath and *ca.* 1 mL of Jones' reagent was added dropwise. After 3 min. the reaction was brought to room temperature and excess reagent decomposed with 2-propanol. The semisolid residue was extracted with pentane (4 × 3 mL) and the combined pentane extract washed with saturated NaCl (1 × 5 mL) and 1 N NaOH (3 × 3 mL). The combined NaOH solution was washed with 5 mL of pentane, acidified with 6 N HCl and extracted with pentane (3 × 3 mL). The pentane extracts were dried and concentrated to a residue (150 mg).

A solution of the residue in CH_2Cl_2 (1 mL) was added to a solution of DCC (250 mg) and *p*-toluidine (120 mg) in CH_2Cl_2 (2 mL). The mixture was stored overnight and filtered. The filtrate was concentrated and the obtained residue (oil) was crystallized from hexane to constant specific activity. The *p*-toluidide showed a [^{3}H: ^{14}C]-ratio of 0.096 compared to 11.84 for the starting alcohol (counted as the 3,5-dinitrobenzoate). This indicates that more than 99% of ^{3}H was located at C-1 of (**2b**).

[1-^{14}*C*]-*Octanol*. The apparatus consisted of a CO_2 generator connected successively to three traps containing ether solutions of heptyl magnesium bromide, a drying tube filled with KOH pellets, and two traps containing 50% aqueous KOH. Barium carbonate (90 mg, 25 mCi) was placed in the generator. A solution of 6 mmole of heptyl magnesium bromide in ether (10 mL) was distributed among the three traps (5 mL/2.5 mL/2.5 mL), and ether added to cover the inlets of the traps. With N_2 flowing the first trap was cooled in ice–acetone and the other two in ice–water baths. The N_2 flow was reduced to a minimum and conc. H_2SO_4 (3 mL) added dropwise to the $BaCO_3$ (30 min). The generator was then heated to 130°, and finally flamed until the solid was completely dissolved. After cooling, the system was flushed with N_2 for 1.5 h.

The contents of the traps were poured into H_2O (30 mL), and the traps were rinsed with 6 N HCl and ether. The mixture was acidified, the ether separated, and the aqueous layer washed with ether (2 × 5 mL). The combined ether solution was washed with 1N NaOH (4 × 15 mL). The combined NaOH extract was washed with ether (2 × 10 mL), acidified (6 N HCl), and extracted with ether (7 × 10 mL). The combined ether extract was washed, dried ($MgSO_4$), concentrated to 5 mL, and ethereal CH_2N_2 added until a yellow color persisted. The solution was concentrated (5 mL), dried ($MgSO_4$), and diluted with ether to 10.0 mL and counted (23 mCi of ^{14}C).

The solution of the methyl octanoate was cooled in an ice bath, and $LiAlH_4$ (25 mg) added in small portions

with cooling. The mixture was refluxed overnight, quenched with H_2O, and 6 N HCl added until the solids dissolved. The product was recovered with ether and processed in the conventional manner to give an ether solution (10 mL) of [1-^{14}C]-octanol (17 mCi).

An aliquot (3.4 μCi) was added to 104 mg of octanol and converted to octyl 3,5-dinitrobenzoate. All of the ^{14}C was retained in the 3,5-dinitrobenzoate ester.

Culture of the organism

Pseudomonas oleovorans strain TF4-1L (Exxon Research and Engineering Co., Linden, NJ) was grown in 15-L batches in an atmosphere of air saturated with *n*-hexane vapor. The cultivation medium contained, per L of demineralized water, KH_2PO_4 (0.2 g), Na_2HPO_4 (0.8 g), $(NH_4)_2$ (1.5 g), yeast extract (Difco, 0.2 g), $Na_2EDTA \cdot 2\ H_2O$ (20.0 mg), $NiSO_4 \cdot 6H_2O$ (0.1 mg), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.1 mg), H_3BO_4 (0.1 mg), $AlK(SO_4)_2$ (0.1 mg), $CuSO_4 \cdot 5\ H_2O$ (0.1 mg), $ZnSO_4 \cdot 7\ H_2O$ (1.0 mg), $CoSO_4 \cdot 7H_2O$ (2.0 mg), $CaCl_2 \cdot 2H_2O$ (1.0 mg), $FeSO_4 \cdot 7H_2O$ (2.0 mg), $MnSO_4 \cdot H_2O$ (5.0 mg), and $MgSO_4 \cdot H_2O$ (30.00). Using 10% inocula, typical yields of 2 g of wet cells per L were obtained after 12–15 h from carboys maintained at 24°.

Enzymic hydroxylations

Two sets of (1*R*)- and (1*S*)-octane incubation experiments were carried out using different batches of *P. oleovorans* strain TF4-1L. Harvested cells were suspended in 50 mM potassium phosphate (pH 7.2) at a mass: volume ratio of 1:1 and homogenized (15,000 psi) in a French pressure cell. Homogenates were centrifuged at 4° (30 min) at 35,000 × g, and the supernates used for hydroxylation of chiral octanes.

An aliquot of supernate (2.5 mL), containing *ca.* 90 mg protein was added to 3 mL of 50 mM potassium phosphate containing 0.09 mmol of 4-phenylsemicarbazide-HCl (pH 6.7) and the mixture shaken for 20 min. Hydroxylation was initiated by the addition of NADH (10 μmol in 0.5 mL of 50 mM potassium phosphate, pH 7.2) and 1 μL (45 μCi of ^{3}H) of *chiral octane* in 20 μL of octanol. Reaction mixtures in tightly sealed screw-cap test tubes were vigorously shaken at 26° for 4 h.

Incubations were terminated by saturating the reaction mixtures with NaCl, and the products were recovered with CH_2Cl_2 (3 × 10 mL). The organic phase was washed with 1 N HCl (2 × 10 mL), H_2O (10 mL), brine (10 mL), and dried ($MgSO_4$). To the dried solution unlabelled octanol (200 μL) was added and the solution was concentrated by distillation through a 20-cm Vigreux column to 1–2 mL. The residue was applied to a column of silica gel (11 g) prepared with pentane and eluates (50 mL) collected. The column was washed successively with pentane (100 mL) and ether–pentane (1:9). An aliquot of each eluate was assayed by gc. Octanol, free of contaminating octane generally appeared in fractions 5 through 7 of ether–pentane (1:9). The octanol-containing fractions were pooled and distilled through a Vigreux column, then transferred to a 5 mL volumetric flask. The flask was filled to mark with pentane, and aliquots (25 μL) were counted. The results are summarized in Table 3. The [^{3}H: ^{14}C]-ratios were determined on the derived octyl-3,5-dinitrobenzoates.

Analysis of biosynthesized octanols

Exchange reactions were carried out in parallel using octanols derived enzymically from (1*R*)- and (1*S*)-octane and synthetic (1*RS*) [1-^{3}H][1-^{14}C]-octanol in 10 mL buffer (77 mM potassium phosphate + 3.4 mg $Na_2EDTA.2H_2O$, pH 8.0) containing ovalbumin (7 mg), NAD (0.62 μ mol), NADH (0.62 μ mol) HLAD (10 mg, 19 U), and porcine heart diaphorase (0.13 mL, 1.3 mg, 180 U assayed as lipoamide dehydrogenase using DL-α-lipoamide).[12,13] The vials were flushed with N_2, and reactions were initiated by injecting the relevant alcohol (20 μL) (see Table 4). Reaction mixtures were vigorously shaken in the dark at 35° for 24 h, then aliquots (1 mL) were withdrawn by syringe and injected into a suspension of NaCl (0.3 g) and octanol (2.5 μL) in ether (5 mL). The suspension was shaken at room temp. (30 min) and the aqueous and organic layers separated by centrifugation. The ether layer was dried ($MgSO_4$) and distilled through a 10-cm Vigreux column to a small volume.

The residue was diluted with dry benzene (3 mL) and 3,5-dinitrobenzoyl chloride (250 mg) added. Following dissolution of the acid chloride, pyridine (2 drops) was added, and the solution refluxed for 90 min. The product was recovered with ether (30 mL) processed in the usual way and the obtained residue was applied to a 20 cm × 20 cm silica gel plate. The plate was developed in benzene–ethyl acetate (150:1), and the band corresponding to octyl-3,5-dinitrobenzoate scraped. The alcohol derivative was eluted from the silica gel with ether, crystallized several times from ethanol and counted.

Octanoic acids from biosynthetic octanols

Biosynthetic octanol (15 μL) in acetone (0.5 mL) was stirred with Jones' reagent (200 μL) at room temperature. The reaction was terminated with 2-propanol. The product was extracted with pentane (4 × 5 mL), the pentane solution washed with brine (2 × 1 mL), and the acidic fraction extracted with 1 N NaOH (4 × 1 mL). The alkaline solution was acidified (conc. HCl) and extracted with pentane (3 × 2 mL). The pentane solution was processed in the usual manner to give an acidic residue. To the residue $SOCl_2$ (0.2 mL) was added and the mixture refluxed (45 min), then more $SOCl_2$ (0.1 mL) added, and refluxing was continued for another 45 min. A solution of *p*-toluidine (100 mg) in dry benzene (1 mL) was added and the mixture refluxed for 30 min.

The reaction mixture was diluted with ether (10 mL), worked up in the conventional manner and the resulting residue applied to a 20 × 40 cm silica gel plate (benzene–ethyl acetate (9:1)). The band corresponding to octanoyl-*p*-toluidide was scraped and the product recovered with ether. Following rechromatography in the same system, the obtained *p*-toluidide was crystallized several times

Table 3. Recovery of octanols from incubations of (1*R*)- and (1*S*)-octane with extracts of *P. oleovorans* strain TF4-1L

EXPERIMENT	CHIRALITY OF OCTANE	[^{3}H:^{14}C] RATIO OF OCTANE	^{3}H-OCTANE INCUBATED	^{3}H-OCTANOL RECOVERED
1	(1R)	8.96	45 µCi	0.56 µCi
	(1S)	8.47	45 µCi	0.75 µCi
2	(1R)	8.96	45 µCi	0.81 µCi
	(1S)	8.47	45 µCi	0.88 µCi

Table 4.

Experiment	Source of Octanol	[^{3}H:^{14}C] Ratio of Octanol *	[^{3}H:^{14}C] Ratio of Derived Octanoic Acid **	dpm of ^{3}H-octanol Used for Equilibration $\times 10^{-3}$	[^{3}H:^{14}C] of Octanol Recovered After 24-h Incubation *
1	(1R)-octane	10.19	7.50	90	8.65
	(1S)-octane	10.10	7.90	125	8.17
	Synthetic (1RS)-octanol	5.76	-	100	2.01
2	(1R)-octane	9.66	6.86	200	7.92
	(1S)-octane	9.07	6.41	200	6.83
	Synthetic (1RS)-octanol	5.98	-	100	1.81

* Counted as the 3,5-dinitrobenzoate ester in 15 ml Liquifluor

** Counted as the p-toluidide in 15 ml Liquifluor
The [^{3}H:^{14}C]-ratios showed an average variation of ± 0.05 on successive crystallization of the derivatives.

from n-hexane and counted. The results are summarized in Table 4.

Calculation of results

100 (% ^{3}H lost in equilibration) = (% lost from R)x_R + (% lost from S)x_S (eqn 1)

x_R = % *R* alcohol in mixture
X_S = % *S* alcohol in mixture
X_R = 100-x_S

During 24 h equilibration (1*R*)-alcohol lost 100% of ^{3}H
During 24 h equilibration, (1*S*)-alcohol lost *Y*% of ^{3}H
Therefore eqn (1) becomes:

100% (% ^{3}H lost in Equil.) = (100 − x_S) + Yx_S (eqn 2)

Determination of Y from racemic octanol

Data: Initial (1*RS*)-octanol [^{3}H: ^{14}C]: 5.98
After 24 h equilibration: 1.81
% ^{3}H lost: 70

Equation (2) becomes:

100 (70) = 100 (100–50) + *Y* (50)
Y = 40% ^{3}H lost from (1*S*)-alcohol

Determination of x_R *and* x_S *for* (1R)*-octane-derived alcohols* (*Expt.* 2)

Data: Initial biosynthetic alcohol [^{3}H: ^{14}C]: 9.66
After 24 h equilibration: 7.92
Octanoic acid [^{3}H: ^{14}C] 6.86

^{3}H available for exchange: 9.66 − 6.86 = 2.80

$$\% \text{ of available } ^3\text{H lost} = \frac{100\,(9.66 - 7.92)}{(9.66 - 6.86)} = \frac{174}{2.80} = 62\%$$

Equation 2 becomes:

100 (62) = 100 (100 − x_S) + 40x_S
x_S = 63%
x_R = 37%

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