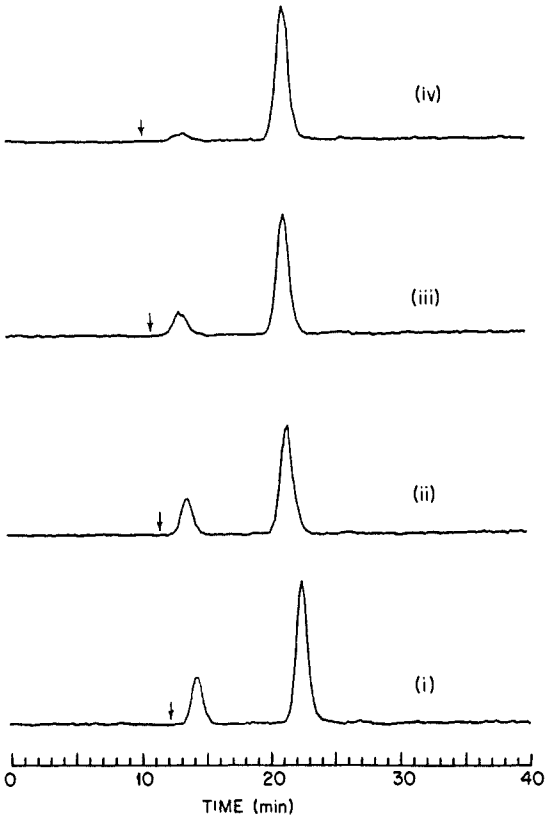

Figure 2. Chromatographic profile of lysozyme as a function of incubation time on the 35μL C4-RP column. Incubation time: (i) 0 min, (ii) 0.5 min, (iii) 1.0 min, (iv) 1.5 min, 5 μg injected, 0.3 mL/min, 4 °C, EX wavelength, 295 nm; EM wavelength, 350 nm; solvent A: 10 mM phosphoric acid, pH 2.3; solvent B: 90% (v/v) methanol in 10 mM phosphoric acid, pH 2.3; gradient: 1–100% solvent B in 10 min. The arrow indicates the time of injection.

over a 6-s period. Kinetic changes were followed from the emission intensity changes as a function of time at a fixed wavelength of 347 nm and a flow rate of 0.3 mL/min. For chromatography, a 10-min linear solvent gradient was employed from solvent A (1% methanol in 10 mM H_3PO_4, pH 2.3) to 100% solvent B (90% methanol in solvent A), both solvents being sparged by helium.

Figure 1A shows the emission spectra (corrected for the blank) of lysozyme adsorbed on the C4-RP surface under solvent A conditions. Spectrum i was collected 15 s after the protein contacted the top of the column and spectrum ii after 90 s. Care was exercised to eliminate photooxidation of Trp. Environmental changes of one or more of the six Trp's (tryptophans) of lysozyme must have occurred upon adsorption, since a fast red shift (solution 346–354 nm) followed by a slower blue shift of λ_{max} (354–349 nm) with an emission intensity increase is observed. These changes clearly arise from the influence of the stationary phase. Figure 1B shows a typical intensity versus time plot obtained at fixed wavelength (347 nm). A least-squares analysis of the plot of $\log(I - I_f)$ versus time (I, intensisty at 347 nm at time t, and I_f, intensity at 347 nm after the blue shift has been completed) yielded a rate constant of 2.7×10^{-2} s^{-1} for the slower step. Figure 2 shows the gradient chromatographic profile as a function of the incubation time of lysozyme on the small C4-RP cell column. Two peaks are observed, the second peak growing at the expense of the first one. The rate constant was determined by the chromatographic method previously described[8] with a value of 1.9×10^{-2} s^{-1}, in close agreement with the fluorescence result.

The emission λ_{max} of the lysozyme solution in solvent A (1% methanol) at 4 °C was 346 nm, in agreement with the data reported in the literature for a pure aqueous phase.[14,15] As soon as the protein adsorbed onto the surface, an 8-nm red shift occurred, indicating that some Trp's of the lysozyme became rapidly exposed to solvent. This result suggests that one or more Trp's in the native protein had become exposed to a more hydrophilic microenvironment. After the first contact of the protein with the surface, the exposed Trp's moved toward a more hydrophobic environment, causing a blue shift in the peak maximum and an increase in the intensity of the fluorescence spectrum.[14-17]

The solvent exposure of Trp residues after the initial contact of lysozyme with the hydrophobic surface is undoubtedly related to a conformational change of the protein. The subsequent conversion, during a period of 2 min, of exposed residues to a more hydrophobic region (most likely contact with the hydrophobic surface) may be due to a further conformational change and reorientation (rearrangement) of the molecule. The agreement between the first-order rate of conversion of lysozyme as measured chromatographically with that measured by surface fluorescence suggests that the two processes have a similar origin. The weaker binding state (i.e., earlier eluting species) can be associated with λ_{max} of 354 nm and the stronger binding state with that of 349 nm. This assignment is reasonable if it is accepted that exposed Trp's contact the *n*-alkyl surface for the later eluting species.

In conclusion, the combination of surface intrinsic fluorescence and chromatography has been shown to provide an effective means of probing the surface dynamics of a protein in contact with an adsorbent. Work is continuing in this area, and full details will be reported subsequently.

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Evidence for a Cytochrome P-450 Catalyzed Allylic Rearrangement with Double Bond Topomerization

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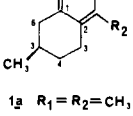
Pennyroyal oil is a volatile plant oil which has been used as an abortifacient.[1,2] However, the high doses required can cause hepatic necrosis and death.[3] Toxicity studies in mice have revealed that (*R*)-(+)-pulegone (5-methyl-2-(1-methylethylidene)cyclohexanone), **1a**, the major constituent terpene of the oil, is re-



1a $R_1 = R_2 = CH_3$
1b $R_1 = R_2 = CD_3$
1c $R_1 = CD_3$, $R_2 = CH_3$
1d $R_1 = R_2 = CD_2H$

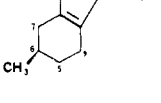


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Table I. Deuterium Incorporation in Pulegone-d_3 and in Menthofuran[a]

m/z	deuteriated form	% of total
	Pulegone-d_3[b]	
153	D_1	1.70
154	D_2	18.30
155	D_3	60.19
156	D_4	18.25
157	D_5	1.57
	Menthofuran[c]	
150	D_0	5.68 ± 0.43
151	D_1	15.42 ± 2.32
152	D_2	20.68 ± 4.93
153	D_3	46.70 ± 4.98
154	D_4	11.52 ± 1.62

[a] Formed by microsomal cytochrome P-450 catalyzed oxidation of pulegone-d_3 as determined by MS and GCMS. [b] Deuterium incorporation was determined by MS on a VG7070H instrument. The sample was introduced via the reference inlet. [c] Deuterium incorporation was determined by GCMS on a VG7070H instrument operating in the selected ion recording mode, by using a 30 m × 0.32 mm DB V capillary GC column. The values are means ± standard deviations for three determinations.

sponsible for the toxicity.[4] Menthofuran (4,5,6,7-tetrahydro-3,6-dimethylbenzofuran) **2** has been identified as a proximate toxic metabolite of (*R*)-(+)-pulegone, and investigations with (*R*)-(+)-pulegone-d_6, **1b**, suggest that it is formed by initial cytochrome P-450-catalyzed oxidation of an allylic methyl group.[5] Metabolic studies with (*R*)-(+)-pulegone-d_3, **1c**, were undertaken to probe further the mechanism of menthofuran formation.

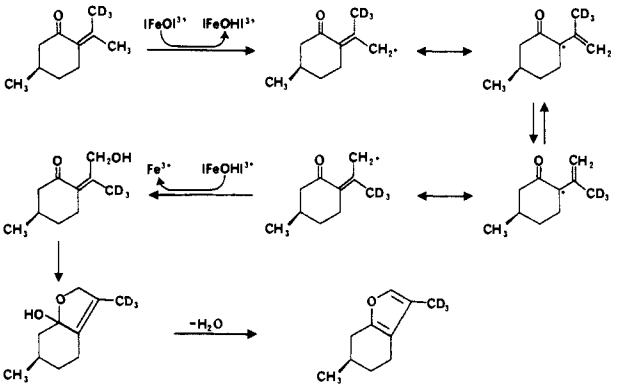
Pulegone-d_3 was synthesized by base-catalyzed deuterium exchange on (*R*)-(+)-pulegone, by using Na_2CO_3 and D_2O in THF. The 300 MHz ^1H NMR spectrum of **1c** in $CDCl_3$ showed the absence of a signal for the (*Z*)-methyl group (δ 1.97 for **1a**) and no detectable decrease in signal intensity for the *E*-methyl group (δ 1.77 for **1a** and **1c**). The 46 MHz ^2H NMR spectrum of **1c** showed only a major signal for the (*Z*)-CD_3 group.

As shown in Table I, menthofuran containing three deuterium atoms was the major metabolic product of **1c**. If oxidation of the (*Z*)-methyl group had occurred, then menthofuran containing one deuterium atom would be expected as the major product. The fact that menthofuran-d_3 is the major product implies that topomerization of the isopropylidene moiety has occurred during the process of furan formation.

A mechanism consistent with thse observations is shown in Scheme I. Cytochrome P-450-catalyzed hydrogen atom removal would occur preferentially from the (*E*)-methyl group because of a deuterium isotope effect. The resulting radical is resonance-stabilized, and rotation about the bond joining the isopropylidene moiety to the cyclohexane ring can occur. The radical may then recombine with the equivalent of hydroxyl radical from the P-450 enzyme, and the primary alcohol could then cyclize and dehydrate to form menthofuran. We have previously shown that the furanyl oxygen is derived from atmospheric oxygen.[5] A minimal value of 72.8 ± 6.6% was calculated for the percentage of menthofuran arising via double bond topomerization by this mechanism.

Additional support for this mechanism was obtained as follows. First, when a 1:1 mixture of *E*:*Z* isotopomers of **1c** (prepared by treating **1c** with CF_3CO_2H in $CDCl_3$) was incubated with mouse liver microsomes, the deuterium content of the resulting menthofuran was the same as that of menthofuran produced from **1c** only. Analysis of the ^1H NMR of pulegone recovered from this incubation showed no detectable change in the isotopomeric ratios of the *Z* and *E* methyl groups. Secondly, a presumed *E*-allylic alcohol was detected by GCMS as another metabolite of (*R*)-(+)-pulegone formed in microsomal incubations.[6] The ratio of the formation rate of this metabolite to menthofuran remained constant at 0.5 in incubations of pulegone and deuteriated analogues thereof. Furthermore, this metabolite did not convert to menthofuran either during the incubations or during the workup procedures.

Scheme I. Proposed Mechanism of Cytochrome P-450-Catalyzed Formation of Menthofuran-d_3 from Pulegone-d_3[a]



[a] The brackets indicate proposed oxidation states for the heme iron of cytochrome P-450.

Thirdly, support is derived from mechanisms proposed for aliphatic carbon hydroxylation by cytochrome P-450.[7,8] Seminal evidence for the radical abstraction–recombination mechanism has come from studies which show large kinetic deuterium isotope effects and significant loss of stereochemistry at the hydroxylated carbon.[9] Although the observed intermolecular isotope effect (k_H/k_D = 1.22 ± 0.10)[10] for the formation of methofuran from pulegone-d_3 (**1c**) was low, the observed intramolecular isotope effect (k_H/k_D = 7.72 ± 0.74)[10] from pulegone-d_4 (**1d**) was significantly higher and consistent with a radical abstraction mechanism. Masking of the intermolecular isotope effect could be caused by the topomerization reaction, substrate binding and dissociation, and/or product release.[11]

Finally, allylic rearrangement of double bonds has been shown to occur during cytochrome P-450-catalyzed oxidation of 3,3,6,6-tetradeuteriocyclohexene, methylenecyclohexene, and β-pinene[12] and the hydroxylation of 3,4,5,6-tetrachlorocyclohexene by rat and housefly microsomes.[13] The novelty of the proposed rearrangement in the pulegone to menthofuran transformation lies in the change in molecular topography that occurs.

Acknowledgment. Financial support from the NIH (2-RO1-GM-25418) is gratefuly acknowledged. The NMR spectrometer used in this work was purchased with funds from the NIH BRSSIG (1-S10-RR-02747-01).

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(6) GCMS analysis was on a VG7070H instrument operated in the scanning mode. GC and MS conditions were as described in ref 5. Retention times of menthofuran and the presumed (*E*)-allylic alcohol relative to the internal standard, 3-methylcyclohexanone, were 1.64 and 2.97, respectively. Consistent with the (*E*)-allylic alcohol structure are ions at *m/z* 168 ($M^{+\cdot}$), *m/z* 150 (M − H_2O), *m/z* 139 (M − HCO), and *m/z* 108 (M − H_2O, $-C_3H_6$). A spectrum and proposed fragmentation pattern are available as Supplementary Material.
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Supplementary Material Available: Experimental and calculational details and ^1H NMR and ^2H NMR spectra for pulegone and pulegone-d_3 (11 pages). Ordering information is given on any current masthead page.

The Acidity of Benzyl Ketones in Aqueous Solution. Evaluation of the Intrinsic Acidifying Effect of a Phenyl Group

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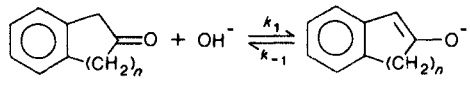
The determination of aqueous pK_a values for aldehydes and ketones has become a subject of much recent interest.[1] Since simple aldehydes and ketones generally have pK_a values of about 15–19,[1c-f] their acidities must be determined indirectly. We now wish to report the *direct* observation of the enolate ions of two substituted cyclic benzyl ketones (2-indanone and 2-tetralone) in aqueous solution. These two compounds are over 10^6-fold more acidic than simple aliphatic ketones and about 10^3-fold more acidic than an acyclic model benzyl methyl ketone.

When 2-indanone (**1**) is dissolved in moderately concentrated (ca. 0.01–1.0 M) hydroxide ion solutions, an intense (ϵ = 16 400 M^{-1} cm^{-1}) peak appears in the ultraviolet spectrum at 288 nm.[2] The high extinction coefficient and relatively long wavelength are consistent with the formation of the anion $\mathbf{1}^-$ (eq 1). Titration



(1)

1, n = 1 **2**, n = 2 $\mathbf{1}^-$, n = 1 $\mathbf{2}^-$, n = 2

of this absorbance as a function of hydroxide ion concentration (10^{-7}–0.67 M, μ = 1.0, 25.0 °C, 4% MeOH) gives a value for the equilibrium constant of 39.0 ± 2.1 M^{-1}, which corresponds to a pK_a of 12.21 ± 0.03.[3] Similarly, 2-tetralone (**2**) exhibits a peak in basic solution at 300 nm (ϵ = 17 300 M^{-1} cm^{-1}), which can be assigned to its anion ($\mathbf{2}^-$). Spectral titration of 2-tetralone gives a pK_a of 12.92 ± 0.02.

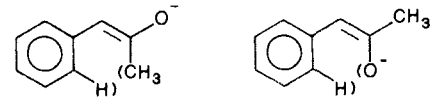
The rate of approach to equilibrium was measured by rapidly mixing 2-indanone with aqueous sodium hydroxide solutions (0.0025–0.03 M, μ = 0.1, 25.0 °C, 1% MeOH). A linear relationship between the observed rate constant and the hydroxide ion concentration (eq 2) allowed the determination of the rate

$$k^{obsd} = k_1[OH^-] + k_{-1} \quad (2)$$

constants for formation of the enolate ion (k_1 = 219 ± 3 M^{-1} s^{-1}) and reprotonation of the enolate ion by water (k_{-1} = 5.88 ± 0.05 s^{-1}). Division of k_1 by k_{-1} yields a value of K = 37.2 ± 0.7 M^{-1}, corresponding to a pK_a of 12.23 ± 0.01, in excellent agreement with the value obtained from spectral titration.

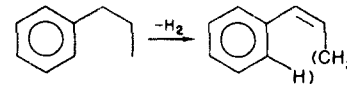
Both **1** and **2** are surprisingly acidic for simple ketones. Acetone (pK_a 19.16)[1d] is 10^7-fold less acidic that 2-indanone and more than 10^6-fold less acidic than 2-tetralone. However, benzyl methyl ketone (**3**, pK_a 15.9),[1b,4] an acyclic model for 2-indanone and 2-tetralone, is only about 10^3-fold more acidic than acetone.

In order to interpret the acidity difference between the cyclic benzyl ketones and benzyl methyl ketone, it is instructive to examine the steric interactions in both the starting ketones and the enolate ions. In the case of benzyl methyl ketone, there are several conformations with little steric interaction between the phenyl group and the rest of the molecule. Delocalization of the negative charge in the enolate requires that the anion be in either conformation **4** or **5**, both of which have significant steric interactions between an ortho hydrogen of the phenyl ring and either the methyl group or the alkoxide oxygen.



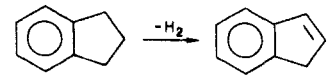
4 **5**

We expect conformation **4** to be preferred due to inhibition of solvation of the negatively charged oxygen in **5** by the ortho hydrogen. An estimate of the magnitude of the steric interaction of the methyl group and the ortho hydrogen in **4** can be obtained by comparing the difference in Gibbs energies of formation of 1-phenylpropane (**6**) and *cis*-1-phenylpropene (**7**) with the difference between indane (**8**) and indene (**9**) (eq 3 and 4).[5] The



ΔΔG_f (3)

	6	**7**	
ΔG_f(g) (kcal/mol)	32.8	51 8	19 0



(4)

	8	**9**	
ΔG_f(l) (kcal/mol)	36 0	51 2	15.2

energy cost of putting a double bond into 1-phenylpropane is ca 3.8 kcal/mol higher than putting a double bond into indane.[7] Thus, benzyl methyl ketone should be ca. 2.8 pK_a units less acidic than 2-indanone, just on the basis of steric factors alone. These unfavorable steric interactions in the enolate of benzyl methyl

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(2) In neutral solution the spectrum of 2-indanone shows peaks at 268 and 275 nm ($\epsilon \sim$ 1000 M^{-1} cm^{-1}).

(3) This pK_a is a concentration equilibrium constant, based upon a value of 1.59 × 10^{14} M^2 for the ion product of water in solutions of ionic strength 0.1.[1d] Additional studies with 2-indanone in ionic strength = 0.1 show that the same value of K is obtained as in solutions of ionic strength = 1.0. Our pK_a value for 2-indanone agrees well with an independent measurement (12.20 ± 0.08) by Keeffe et al. (Keeffe, J. R.; Kresge, A. J.; Yin, Y., following paper in this issue).

(4) Benzyl methyl ketone gives no observable spectral change between pure water and 1.0 N sodium hydroxide solutions, consistent with Guthrie's[1b] previous estimate of pK_a 15.9.

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