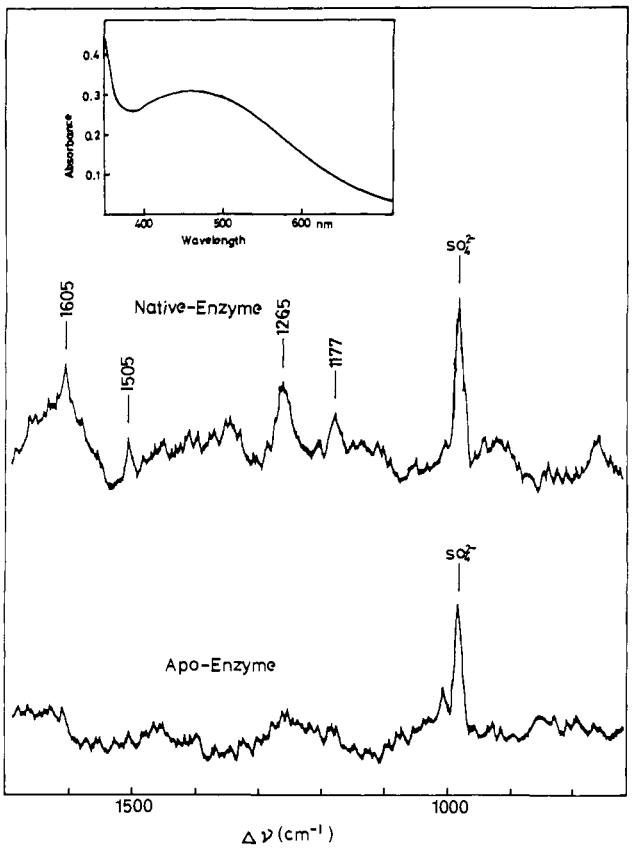

Figure 1. Raman spectra of protocatechuate 3,4-dioxygenase. Instrumental conditions: excitation, 488.0-nm line of Ar^+ laser; power, 70–140 mW at a sample point; time constant, 16 s; slit width, 180 μm; scan speed, 10 cm^{-1}/min; temperature, 15 °C. Concentrations: 32 and 29 mg/mL for the native and apo enzyme, respectively, in 50 mM of Tris-acetate buffer, pH 8.5. The inset shows the visible absorption spectrum of protocatechuate 3,4-dioxygenase, 23.3 mg, in 2.8 mL of 50 mM Tris-acetate buffer, pH 8.5.

technique is known to give structural information on chromophores,[6,7] we applied it to this enzyme.

The crystalline native enzyme and its apo enzyme were prepared as described previously.[8] As the intense visible absorption band at ~450 nm (ϵ 2.6 × 10^4 M^{-1} cm^{-1}) is associated with a charge transfer from the ligand to metal, a ligand-iron stretching mode or some internal vibrations of the ligand may gain resonance Raman intensity upon excitation at wavelength near the 450-nm band. Thus the Raman spectra were excited by the 488.0-nm line of an argon ion laser (Spectra Physica Model 164) and were recorded on a JEOL-400D Raman spectrometer equipped with HTV-R649 photomultiplier.

Figure 1 shows the resonance Raman spectra of the native and apo enzyme in the presence of 1% $(NH_4)_2SO_4$ as an internal reference. Four prominent Raman lines were observed at 1177, 1265, 1505, and 1605 cm^{-1} for the native enzyme, but none of these lines was detectable for the apo enzyme and the colorless ferrous form of enzyme prepared from the native enzyme by reducing with $Na_2S_2O_4$ under anaerobic conditions. Thus, the appearance of these Raman lines apparently requires the presence of ferric iron. These lines appear to be in resonance with the visible absorption band, and are presumably due to the internal vibrations of the coordinated amino acid residue. Their frequencies were unaltered in D_2O solution, indicating that the residues involved in the appearance of the Raman lines contain no replaceable hydrogen. The Raman spectrum of *p*-cresol-iron(III) complex prepared by mixing ferric ammonium sulfate with *p*-cresol at pH 7.0 showed four lines at 1180, 1222, 1488, and 1618 cm^{-1}. *p*-Cresol in 1 M NaOH solution also gave the corresponding Raman lines at 1176, 1276, 1490, and 1607 cm^{-1} with relative intensities different from those of the enzyme. These spectral data resemble those of iron(III)-transferrin reported by Gaber et al.,[9] which show four prominent Raman lines at 1174, 1288, 1508, and 1613 cm^{-1}. They assigned those lines to the vibration of phenolate ion of the coordinated tyrosine residue, based on study of a bis phenolate-iron(III) complex.

The present Raman spectrum is thus explicable in terms of the internal vibration of a coordinated phenolate anion of tyrosine residue. The four characteristic Raman lines of the native enzyme remained unshifted in the ES complex though with different relative intensities. This suggests a little conformational change at the active site and retention of the coordinated tyrosine upon substrate binding. Upon coordination of a cysteinyl sulfur to Fe^{3+} ion the Raman line due to Fe^{3+}-S stretching modes is expected to appear in a region between 250 and 350 cm^{-1} as seen in iron-sulfur proteins.[10] Despite a careful search, the corresponding line was not observed. This does not, however, warrant the conclusion that cysteinyl sulfur is not coordinated to the ferric iron of this enzyme.

The present work thus provides an example of successful application of the resonance Raman spectroscopy to the structural studies of such a giant molecule as nonheme iron containing dioxygenases. Further detailed studies of several nonheme iron containing dioxygenases are in progress.

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Catalytic Hydrolysis of Phenyl Esters in Aqueous Didodecyldimethylammonium Vesicles: Remarkable Rate Difference between Intra- and Intervesicle Reactions

Sir:

Stable, well-organized aggregates are formed in water from a variety of dialkylammonium and related compounds.[1-3]

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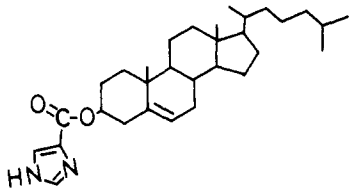
Table I. Effect of Sonication of Mixed Stock Solutions on k_{obsd}[a]

	k_{obsd}, s^{-1}			
	$2C_{12}N^+2C_1Br^-$		CTAB	
	PNPA	PNPP	PNPA	PNPP
Series A[b]	0.53	0.032	0.066	0.12
Series B[c]	0.45	7.8	0.065	0.19

[a] Substrate, (1.2-1.5) × 10^{-5} M; catalyst, (1.1-1.2) × 10^{-4} M except for 1.1 × 10^{-5} M in the hydrolysis of PNPP in series B. The observed rate constants were corrected to the values for [catalyst] = 1.0 × 10^{-4} M. $2C_{12}N^+2C_1Br^-$, 1.0 × 10^{-3} M; CTAB, 2.0 × 10^{-3} M. Conditions: 30 °C, pH 9.5 ± 0.1, 0.01 M borate buffer; μ = 0.01 (KCl). [b] Simple mixing of stock solution. [c] Sonication of mixed stock solutions.

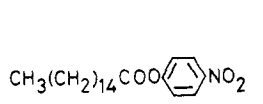
NMR and ESR studies indicate that these aggregates possess highly viscous organic phases which may give rise to the phase transition between solids and liquid crystals.[4,5] These properties are quite different from those of the fluid hydrophobic core of the conventional surfactant micelles.

In this communication, we wish to describe a remarkable rate control by dialkylammonium vesicles in the catalytic hydrolysis of phenyl esters. The rate acceleration effect of these vesicles has been observed in related systems.[6] The catalyst

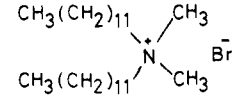


catalyst

used in this study is the cholesteryl ester of imidazolecarboxylic acid, mp 225-230 °C,[6] and the substrates are *p*-nitrophenyl acetate (PNPA), mp 78 °C, and *p*-nitrophenyl palmitate (PNPP), mp 63-65 °C.[7]



PNPP



$2C_{12}N^+2C_1Br^-$

Didodecyldimethylammonium bromide ($2C_{12}N^+2C_1Br^-$), mp 55-56 °C, and substrate were directly sonicated (Bransonic Sonifier 185) in acidic water for 10-15 min to give clear stock solutions. The stock solution of catalyst was similarly prepared in the presence of $2C_{12}N^+2C_1Br^-$. The reaction was initiated by adding 0.1 mL of these stock solutions to 3.3 mL of alkaline buffer solutions (0.01 M borate, μ = 0.01 (KCl))[8] and monitored using the absorption of *p*-nitrophenolate anion at 401 nm. Unless stated otherwise, pseudo-first-order kinetics were observed to at least 80% completion of the reaction. A correction was made for the rate of spontaneous hydrolysis. The turnover of the catalyst occurs in the presence of excess substrates. The final concentration of $2C_{12}N^+2C_1Br^-$ was 1 mM. This solution has been shown to contain multiwalled bilayer vesicles by electron microscopy.[1]

Table I summarizes two series of the experiment. In series A, the two stock solutions were mixed with the buffer solution without sonication. In the second series (series B), the stock solutions were mixed, sonicated for 30-60 s, and then added to the buffer solution. Similar experiments were performed by using micellar hexadecyltrimethylammonium bromide (CTAB) in place of $2C_{12}N^+2C_1Br^-$ vesicles. The pseudo-first-order rate constant, k_{obsd}, for the hydrolysis of PNPA in the presence of $2C_{12}N^+2C_1Br^-$ is fairly constant, regardless

Table II. Variation of k_{obsd} with Progressive Ageing of the Stock Solutions[a]

Order of ageing	Ageing condition		10^4k_{obsd} after progressive ageing, s^{-1}
	°C	min	
1	0	30	24
2	21	30	91
3	61	10	640
4	0	30	690

[a] Reaction condition: pH 9.3, 30 °C; catalyst, 1.18 × 10^{-6} M; PNPP, 1.50 × 10^{-5} M; $2C_{12}N^+2C_1Br^-$, 1.00 × 10^{-3} M. The stock solutions of catalyst and substrate were mixed *without sonication,* and subjected to ageing.

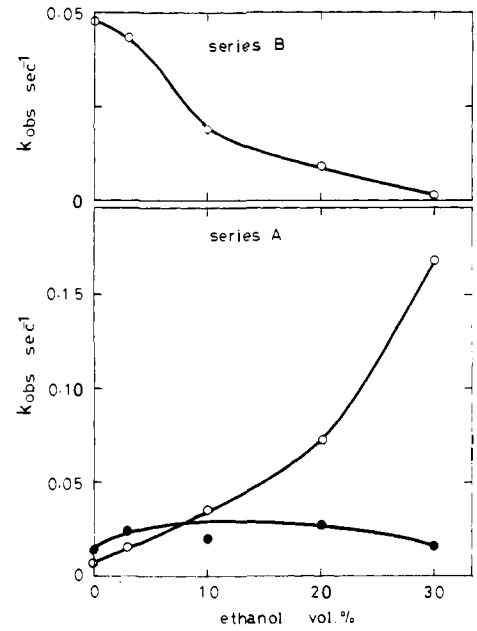


Figure 1. Effect of the solvent composition (ethanol-water) on k_{obsd}: ○, $2C_{12}N^+2C_1Br^-$; ●, CTAB. Substrate: 1.8-3.3 × 10^{-5} M. Catalyst: series A, 1.2 × 10^{-5} M; series B, 2.6 × 10^{-6} M. Condition: pH 9.5 ± 0.3, 30 °C, 0.01 M borate, μ = 0.01 (KCl).

of whether or not a mixture of the stock solutions is sonicated. In sharp contrast, the rate of the catalytic hydrolysis of PNPP is quite different between the two series of the experiment. By sonication of the mixed stock solution, k_{obsd} is enhanced by a factor of over 200. The catalytic hydrolysis of PNPP is much slower than that of PNPA without sonication of the mixed stock solution, but is much faster with sonication. When CTAB was used in place of $2C_{12}N^+2C_1Br^-$, the rate difference was nonexistent or small.

The aggregate weight of the vesicle was estimated to be ~0.7 million daltons.[1] Both catalyst and PNPP are insoluble in water and must be completely incorporated into these ammonium aggregates. On average, one vesicle would contain 15-150 catalyst molecules and 30 substrate molecules after complete equilibration by sonication. Therefore, in series B experiments, PNPP must be hydrolyzed exclusively by catalyst molecules in the same vesicle, and the large k_{obsd} difference for PNPP between series A and B experiments (0.032 vs. 7.8 s^{-1}) is derived from the rate difference between the *inter*vesicle and *intra*vesicle reactions. On the other hand, distribution of catalyst and substrate among aggregates reaches equilibrium rapidly in the CTAB system, and the rate difference between the two series of the experiment is negligible. PNPP is hydrolyzed faster than PNPA probably because of better binding.

The effect of heat treatment of the vesicular stock solutions is consistent with the above-mentioned results. In the series A experiment (intervesicle reaction), the reaction rate was not

affected by the temperature of ageing (0 and 50 °C) prior to mixing of the two stock solutions. On the other hand, the temperature of ageing after mixing of the two stock solutions influenced the reaction rate. Table II shows the influence of the ageing procedure on the rate constant.[10] k_{obsd} increases from 0.0024 to 0.0091 s^{-1} when the mixed stock solution was kept at 21 °C for 30 min after having been kept at 0 °C. Further ageing at 61 °C (10 min) raised k_{obsd} by a factor of seven, but additional ageing at 0 °C did not change k_{obsd}. Since the hydrolysis starts only when pH of the reaction medium is adjusted to alkaline by buffer, the results of Table II are explained by redistribution of catalyst and substrate molecules among vesicles during ageing at higher temperatures. The ageing at 0 °C does not seem to promote the redistribution. This is consistent with the phase transition temperature of aqueous $2C_{12}N^+2C_1Br^-$ (13 ± 2 °C) as determined by differential scanning calorimetry.[11]

Figure 1 shows the influence of the solvent composition (aqueous ethanol) on k_{obsd}. In series A experiment (intervesicle reaction), k_{obsd} increased with increasing ethanol contents. The intervesicle reaction is facilitated because ethanol loosens the vesicle structure. In the intravesicle reaction where distribution of the reacting species is completely equilibrated (series B), ethanol rather diminishes k_{obsd}. The latter trend is similar to that observed for the CTAB system in the series A experiment.

In conclusion, the rate difference between intervesicle and intravesicle reactions can be made very large by selecting appropriate reactants which tightly bind to vesicles. The conventional micellar system is too soft for this purpose. The present system may find many interesting applications.

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Detection of ^{13}C–^{15}N Coupled Units in Adenine Derived from Doubly Labeled Hydrogen Cyanide or Formamide

Sir:

The formation of the nucleic acid base, adenine, by a simple heating procedure involving formamide and hydrogen cyanide[1] is of interest from the viewpoint of chemical evolution.[2] Although the formation of adenine from hydrogen cyanide has been reported and reaction mechanisms proposed,[3] there has been no direct experimental evidence as to the mechanism. Feeding experiments with doubly enriched precursors have been successfully applied to solve detailed reaction pathways during the biosyntheses of natural products.[4]

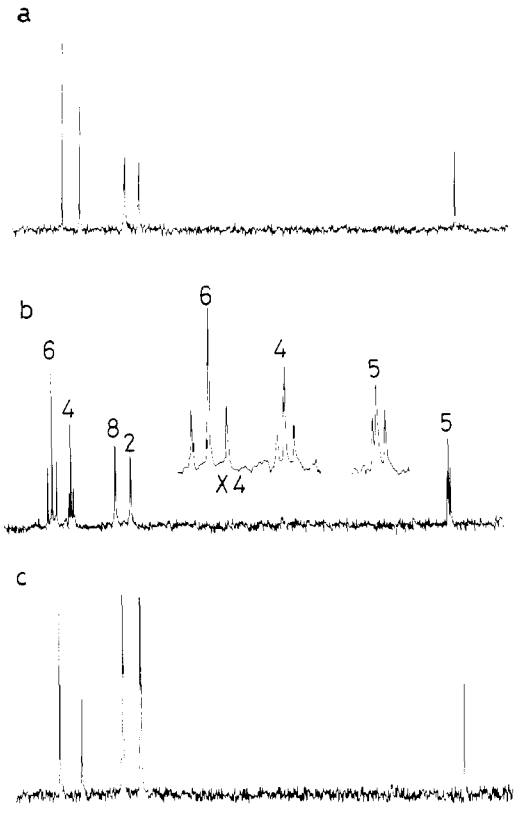

Figure 1. ^{13}C NMR spectra of labeled adenines. Adenine was dissolved in acidified Me_2SO solution (0.5 mL) with 1 drop of concentrated HCl. The spectra were recorded on a JEOL FX-100 NMR spectrometer equipped with a JEOL JEC-980B computer for Fourier transform operation at 25.05 MHz (data points, 4K; spectral width, 1.5 KHz; flip angle, 36°; 3 s between pulses) in a proton-noise decoupled mode: (a) adenine of natural abundance (11 mg), 20 000 pulses; (b) labeled adenine with $H^{13}C^{15}N$ (10.8 mg), 20 000 pulses; (c) labeled adenine with $H^{13}CO^{15}NH_2$ (8.3 mg), 30 000 pulses.

We wish to report the result of ^{13}C NMR experiments on the nucleic acid base, adenine, which was obtained from doubly enriched hydrogen cyanide or formamide.

Doubly enriched potassium cyanide (^{13}C, 90.5%; ^{15}N, 99.2%) was diluted fiftyfold with potassium cyanide of natural abundance (3.75 g) to differentiate newly formed C–N bonds from the labeled C–N bond. Hydrogen cyanide which was generated by acidifying the potassium cyanide with concentrated sulfuric acid was introduced into formamide (10 g) under ice cooling. The mixture was sealed and heated at 160 °C for 5 h. Adenine was extracted with and recrystallized from hot water.[1] ^{13}C NMR spectra of adenine were obtained in acidified Me_2SO-d_6 to improve the peak heights of nonprotonated carbons (C_4, C_5, and C_6.)[5] The chemical shifts of the five carbons of adenine in acidified Me_2SO solution (Figure 1a) were different from those in neutral Me_2SO solution (in parentheses) as follows:[6] C_2, 141.8 (152.2); C_4, 147.4 (151.1); C_5, 112.4 (117.3); C_6, 149.2 (155.1); and C_8, 143.2 (139.2) (parts per million downfield with respect to Me_4Si). Three ^{13}C–^{15}N coupled units were observed in the ^{13}C NMR spectrum of the product, adenine, derived from doubly labeled hydrogen cyanide (Figure 1b). Those are C_4 (J = 9.5 Hz), C_5 (J = 7.3 Hz), and C_6 (J = 20.5 Hz) and are shifted slightly to higher field (0.4, 1.5, and <0.1 Hz, respectively) than those of noncoupled peaks due to ^{15}N isotope shift.

On the other hand, when adenine was prepared by heating doubly enriched formamide (^{13}C, 91%; ^{15}N, 96%, 77 mg was

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