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Diffusion in Rigid Bilayer Membranes. Use of Combined Multiple Pulse and Multiple Pulse Gradient Techniques in Nuclear Magnetic Resonance

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Abstract: Combined NMR multiple pulse homonuclear decoupling and multiple pulse gradient techniques have been used to determine the self-diffusion coefficients at 25 °C of the phospholipids in the $L_{\beta'}$(gel) phase of L-α-dipalmitoylphosphatidylcholine in a 15% (w/w) D_2O model membrane and of potassium oleate in a 30% (w/w) D_2O lamellar phase (above the phase transition). The values found, 1.6×10^{-10} and 1.3×10^{-8} $cm^2\ s^{-1}$, respectively, are considered in reasonable agreement with values obtained by other investigators using fluorescence photobleaching recovery. The technique has the advantage that it monitors lipid protons and hence avoids possible complications of added probes. The use and limitations of the method are discussed, and the values found for diffusion are compared with those determined or estimated by other methods.

The importance of lateral diffusion of lipids, proteins, and other membrane components is well established.[2,3] Diffusion is a fundamental aspect of embryological development, cell fusion, membrane phase separations, membrane transport processes, and immunochemistry.[3] Lipid water multibilayer systems, such as L-α-dipalmitoylphosphatidylcholine (DPL)/H_2O system, have been extensively studied as models of cell membranes.[2c] Such systems, lyotropic liquid crystals, have the capability of maintaining orientational order while allowing for flow.[2c] One of the most useful parameters used in inferring microscopic details about the dynamics of flow in such systems is the self-diffusion coefficient. While pulsed NMR spin–echo methods have been a traditionally useful tool for determining self-diffusion in liquids,[4] relaxation due to dipolar interactions generally precludes their use in solids and in relatively rigid phases such as glasses and model membrane multibilayers. In order to apply this NMR technique in such systems, the dipolar broadening must be removed in some fashion. One method of achieving this, applicable to the special case of membranes where the lipids are above the chain melting phase transition (T_c), is to use oriented samples and the angular properties of the dipolar Hamiltonian.[5,6] Another, and much more general, approach is to utilize the multiple pulse techniques developed in the early 1970s to attenuate dipolar

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broadening in rigid systems.[7-9] This development in turn pointed to combined multiple pulse NMR–pulsed gradient techniques to measure diffusion in systems where dipolar relaxation was a major contributor to the line width.[10] This early work,[10] however, suffered from two problems. The first was that the homonuclear decoupling scheme used did not strongly attenuate dipolar broadening. In addition, field gradient strengths sufficiently strong to allow for measurements of diffusion constants much smaller than 10^{-7} cm^2 s^{-1} were not generally available. Lowe[11] has since developed a method to generate pulse field gradients as strong as 10^3 G cm^{-1} using gradient coils which are sufficiently small in diameter to produce the required intensities with relatively modest power requirements. Use of such geometries, however, limits sample size and thus the signal to noise ratio in a given experiment. In addition, the problem of capacitive coupling between radio-frequency and pulse gradient coils is increased in such geometries, thus making the multiple pulse experiment more difficult. In this report, we use a combined technique which allows for efficient pulse homonuclear decoupling, allows for modest requirements on power and space via the use of multiple pulsed field gradients,[12] allows the use of standard 5-mm NMR sample tubes in which to contain samples, and allows the determination of self-diffusion constants of the order of 10^{-11} cm^2 s^{-1}. In particular we have used this method to measure diffusion of lipids in model membrane lamellar phases below the phase transition, an important quantity[3] for which only a few measurements exist from the use of fluorescent labeled probes.

Experimental Section

The NMR spectrometer operates at 55.7 MHz for proton resonance. With the exception of the units mentioned below it has been previously described.[15] The pulse programmer was designed in this laboratory and is described in detail in a separate paper.[16] The unit has four channels with analogue control of pulse widths, three channels with digital control of pulse widths to 0.1-μs resolution, and three channels used as triggers for experimental events. It is based on a 6800 microprocesser system and pulse sequences are called either from canned programs in ROM or can be entered at the terminal, which in this case is a DECWRITER II.

The quadrupole gradient coil was constructed according to the design of Webster and co-workers.[17] The gradient coil was 4 cm long, had an average i.d. of 15 mm, and was wound with ten turns per quadrant. The total inductance was 4 μH. The coil constant, calibrated by the method of Tanner and Stejskal[4] using the diffusion coefficients of water and glycerol as standards (taken to be 3.2×10^{-8} cm^2/s for glycerol[18] and 2.35×10^{-5} cm^2/s for water[19]), was 9.35 (G/cm·A).

The pulse gradient unit was of our own design, but similar in design to that given in ref 19b. Gradients of 100 G cm^{-1} were achieved in gradient pulses 20 μs in duration.

The combined radio frequency pulse-gradient pulse sequence used in the present work is diagrammed in Figure 1. In this experiment, a relatively weak (for the magnitude of diffusion constants under consideration) gradient pulse $\vec{G}$ of width δ is produced in the 2τ windows between the first and second halves of the MREV-8 sequence for a variable number of times n to produce a "multiple pulse-multiple gradient" (MP-MG) sequence. Subsequently the MREV-8 sequence was produced m times, followed by a π_y pulse in the 2τ window between MREV-8's. The time between the P_x (90° along phase x) preparation pulse and the inverting π_y pulse is τ, determined by the sum $(n + m)$, which is maintained constant. The effective strength of the gradient pulse is determined by the number of gradient pulses n and was typically of order of 10^3 G cm^{-1}. The size of the effective gradient varied by changing n/m such that $(n + m)$ was constant. For effective averaging of homonuclear dipolar broadening it is necessary that multiple pulse cycle times be short compared to the inverse of the dipolar line width.[20] In the present case, with natural line widths less than 323 Hz, cycle times of 120 μs were more than sufficient for narrowing. Following the π_y inverting pulse, the same $n + m$ sequences of MPMG and MREV-8 are used, followed by 2048

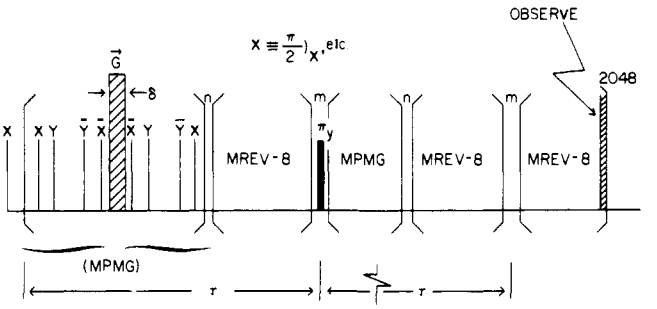

Figure 1. The multiple pulse homonuclear decoupling-multiple pulse field gradient experiment. Pulsed field gradients are inserted in the 2τ windows of the homonuclear decoupling sequence in which the magnetization lies along the same axis as after the rf preparation pulse. Effective gradient strengths are altered by varying the ratio of n/m, keeping $n + m$ constant. Typical values are $(n + m) = 120$, $n = 100$.

Table I. Relaxation Data

sample	T_{1y}, s	T_2*, ms	T_2 (MREV-8), ms
DPL/15% D_2O	0.83	3	7.45
KO/25% D_2O	0.103	5.4	14.5

MREV-8 sequences. Data was accumulated in the 2τ windows following the last 2048 sequences of MREV-8.

The lipid samples were heated to 100 °C and dried for several hours under a pressure of less than 10^{-4} Torr. Then, doing all procedures under dry N_2, they were mixed with the appropriate amount of D_2O at 60 °C (centrifugation through a constriction[5a-c]) and sealed in a 5-mm NMR tube. Under the experimental conditions the samples, essentially liposomes, were stable and gave reproducible diffusion coefficients for periods of several weeks.

The two samples used in the present work were DPL/15% D_2O (w/w) and the potassium salt of oleic acid (KO)/25% D_2O (w/w). The temperature of the sample was controlled to 25 ± 1 °C by air flow through an annular "race track" groove[21] machined in a cylindrical aluminum block in which were inserted the quadrupole and NMR coils. The sample temperature was monitored by a chromel-alumel thermocouple inserted in water in an NMR tube in the sample coil. Temperatures were determined under steady-state pulsing conditions used in the diffusion measurements.

The effective transverse relaxation time, T_2*, was determined from the decays under a single pulse experiment. The transverse relaxation time under the multiple pulse experiment, T_2(MREV-8), was also determined from the decays under that sequence. The longitudinal relaxation time under the multiple pulse sequence,[22] T_{1y}, was obtained from the decay under the 17-pulse experiment consisting of two MREV-8 pulse sequences interspersed by a 180° pulse along y in the rotating frame. Under this sequence, relaxation is due to second-order dipolar terms, cross terms between pulse imperfections and the dipolar Hamiltonian, and the longitudinal relaxation under the multiple pulse sequence. Longitudinal relaxation times of protons in both samples under a single pulse experiment were long compared to the values in Table I.

Analysis of Technique

The attenuation of amplitudes of the spin-echoes in the presence of a time-independent gradient is given for the normal echo technique[4] by

$$A(G)/A(0) = \exp\{-\gamma^2 D(\tfrac{2}{3}\tau^3\vec{G}_0^2 + \delta^2(\Delta - \delta/3)\vec{G}^2 - \delta(t_1^2 + t_2^2 + \delta(t_1 + t_2) + \tfrac{2}{3}\delta^2 - 2\tau^2)\vec{G}\cdot\vec{G}_0)\} \quad (1)$$

where D is the translational self-diffusion coefficient. $A(G)$ is the echo amplitude in the presence of a gradient, and $A(0)$ is the amplitude with $\vec{G} = 0$. The conditions under which this result obtains are that the steady gradient ($\vec{G}_0$) is present at all times and two gradient pulses ($\vec{G}$) of duration δ separated by a time Δ are applied to the system, separated by a refocusing π pulse. As $\vec{G}_0$ approaches zero, only the term in $\vec{G}^2$ remains, with the result

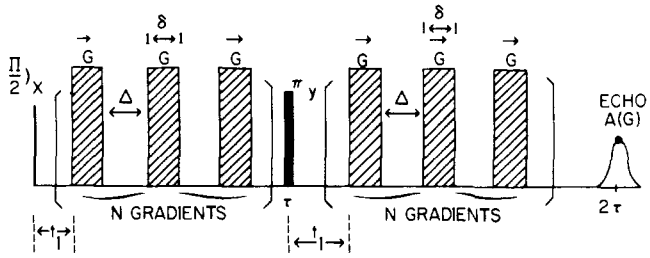

Figure 2. Replacement of a single pulse gradient, G, with N gradients results in an effective gradient NG.

$$A(G)/A(0) = \exp\{-\gamma^2 D\delta^2\vec{G}^2(\Delta - \delta/3)\} \quad (2)$$

In the present work, a single, large gradient pulse is replaced by several smaller pulses[12] prior to the 180° rf pulse and prior to observing the echo, for the purpose of increasing the effective strength of the gradient applied to the sample (G/cm). The attenuation of amplitude for this case without multiple rf pulses between the gradients is given by

$$A(G)/A(0) = \exp\{-\gamma^2 D(\tfrac{2}{3}\tau^3\vec{G}_0^2 + \delta^2 n(\Delta - \delta/3)\vec{G}^2 - 2\delta(\delta n\Delta + \Delta n t_1 + \tfrac{1}{3}n\Delta^2 - n\tau\Delta)\vec{G}\cdot\vec{G}_0)\} \quad (3)$$

where n is the number of gradient pulses applied and Δ, t_1, and τ are indicated in Figure 2, which shows the multiple gradient experiment in the absence of multiple pulse decoupling. The combined technique used in this work represents a superposition of a MREV-8[8,9] multiple pulse sequence, averaging homonuclear dipolar interactions and a multiple pulsed linear magnetic field gradient sequence combined with the Carr-Purcell sequence. The detailed pulse sequence is shown in Figure 1. It consists of a P_x preparation pulse, followed by a train of MREV-8 multiple pulse sequences with a variable number of gradient pulses (n) in the 2τ window between four pulse cycles, followed by a variable number (m) of eight pulses cycles such that $n + m$ = constant. The effective imposed gradient is adjusted by varying n/m. Under the multiple pulse homonuclear decoupling scheme used in the present work, with observation of responses in windows which are multiples of the multiple pulse cycle times, interactions may be represented in average Hamiltonian formulation:[7]

$$\overline{H} = \sum_{i=0}^{\infty} \overline{H}^{(i)} \quad (4)$$

In particular for static dipolar interactions under the MREV-8 sequence

$$\overline{H}_D^{(0)} = \overline{H}_D^{(1)} = 0 \quad (5)$$

so that static dipolar contributions only contribute to second order. An upper limit of this contribution is indicated by the values of T_{1y} in Table I. Offset Hamiltonians, which include the imposed carrier resonance offset, $\Delta\omega$, and the chemical shift, δ, become

$$\overline{H}_{\text{offset}} = \frac{1}{3}\sum_k (\delta_k + \Delta\omega)(I_z + I_y) \quad (6)$$

The imposed pulse field gradient is an offset Hamiltonian, only acting in the 2τ windows between multiple rf pulse sequences:

$$\mathcal{H}_G = -\Delta\omega_G I_z \quad (7)$$

where[23]

$$\Delta\omega_G = \gamma(\partial H/\partial z)\vec{z}(t) \quad (8)$$

for positive gradients. Here, $\vec{z}(t)$ is the effective distance of diffusion in time t over which a shift in Larmor frequency can take place.

If the gradient pulses are square, are equal to unity when the gradient is on, and are zero otherwise, then

$$H_G = \gamma G\vec{z}(t) \quad (9)$$

The dephasing angle of a particular nucleus will be $\omega t = \phi$. From average Hamiltonian theory

$$\overline{H}_G^{(0)} = \frac{1}{tc}\int_0^{tc} \mathcal{H}_G(\vec{z},t)dt \quad (10)$$

Again, as with the simple Carr-Purcell[4] experiment, if the spins move to a region of different field strength between the preparation 90° pulse and the rephasing 180° pulse, refocusing will be incomplete, causing an echo of lower amplitude. The phase angle of each spin is calculated as follows:

$$\phi_s = \gamma\int_0^{\tau} \mathbf{g}\cdot\vec{\mathbf{z}}\,dt - \gamma\int_{\tau}^{2\tau} \mathbf{g}\cdot\vec{\mathbf{z}}\,dt \quad (11)$$

where $\vec{\mathbf{z}} = (z\vec{\mathbf{k}}, 0, 0)$, the distance of diffusion.

The resulting echo attenuation will be the average of $\cos\phi_s$ over all the spins, which for a distribution of phase angles Gaussian about $\phi_s = 0$ is readily shown to be $(-\frac{1}{2}\langle\phi_s^2\rangle)$. Applying the method of calculations of Stejskal and Tanner,[4] Carr-Purcell,[4] and Williams[24] et al., we obtain the following expression for the amplitude of the echo at time = 2τ:

$$A(G)/A(0) = \exp\{-\tfrac{1}{2}\langle\phi_s^2\rangle\}$$

or,

$$A(G)/A(0) = \exp\left\{-\gamma D\left(\int_0^{2\tau}\left[\int_0^{t'}\vec{G}(t'')dt'\right]^2 dt'\right)\right\} \quad (12)$$

This derivation assumes single gradient pulses which may be varied either by a change in intensity or a change in width for fixed intensity. In the present experiment, we replace this single intense gradient application by a number of weaker, more controllable gradient pulses. It is important to understand that these multiple gradient pulses are only equivalent to a single gradient–180° pulse–single gradient experiment *if the multiple gradients are located in a window of the multiple pulse experiment where the magnetization is along the same axis as after the P_x preparation pulse.* In the present case, this means the multiple gradients must be applied in the 2τ windows between the four pulse cycles of which the MREV-8 pulse cycle is composed, or in the 2τ windows between the MREV-8 pulse cycles. In the latter case, under the combined multiple pulse homonuclear decoupling–multiple pulse gradient experiment, the amplitude of the echos obeys the relation

$$A(G)/A(0) = \exp\left\{(-2\tau/T_2(\text{MREV-8})) - \frac{2}{3}\gamma^2\tau\delta^2\vec{G}\cdot\vec{D}\cdot\vec{G}\right\} \quad (13)$$

Here T_2(MREV-8) is the transverse relaxation time under the multiple pulse experiment.

It is well known[2a,b,25,26] that lateral diffusion in membranes is much faster than the almost negligibly slow "flip-flop" diffusion. The samples are very viscous, with a visual appearance perhaps best described as similar to white silicone stopcock grease. Under these conditions, very little motion of the extended liposomes is expected, and any diffusion occurring is almost certainly lateral diffusion.

Experimental Results and Discussion

Equations 12 and 13 for the echo amplitudes were verified experimentally by measurements on water and glycerol at 25 °C. The data taken to obtain self-diffusion constants found for DPL/15% D_2O at 25 °C (the gel phase) and KO/25% D_2O at 25 °C (the high-temperature phase) are indicated in Figure 3. The values found for D are as follows, within our estimated error of ≈50%:

DPL/15% D_2O $D = 1.63 \times 10^{-10}$ cm^2 s^{-1}

KO/25% D_2O $D = 1.3 \times 10^{-8}$ cm^2 s^{-1}

For KO, which at 25 °C is above T_c, our result is in agreement with values measured in one of these laboratories[5d] with the oriented sample NMR technique applied at 30 °C to KO/D_2O samples from which the H_2O had been carefully removed. ESR spin-label results of diffusion of KO[26b] also give values near 10^{-8} cm^2/s.

For lipid lateral diffusion above T_c an abundance of studies with a variety of techniques[5,6,13,14,25-30] yields values of D in the range of $\approx 5 \times 10^{-8}$ cm^2/s for DPL at full hydration (≈40% water) at temperatures just above T_c (≈41 °C), the precise value depending upon the extent of hydration,[5b,c] on the form of the sample (whether oriented multibilayers, vesicles, or single bilayers),[27] and in some systems on the sample preparation technique.[27]

In contrast to the extensive literature on the higher temperature phase, only a few estimates exist of lipid D below the transition. The bulk of these have come from fluorescence photobleaching recovery (FPR) experiments,[14,27,30] although estimates have also been derived from ^{31}P NMR line widths[6d] and from bimolecular kinetics of triplet–triplet annihilation of the triplet states of fluorophore labeled lipids in DPL vesicles.[28] The results may be summarized as follows. FPR data in oriented multibilayers, liposomes, and vesicles are in general agreement that D below the phase transition is of the order of 10^{-10} cm^2/s, about two orders of magnitude less than that above the transition. With FPR in oriented multibilayer DPL systems, Wu et al.[30] determined an upper bound on D of $\lesssim 5 \times 10^{-10}$ cm^2/s at 38 °C, Smith and McConnell[14] measured $D \approx 1.6 \times 10^{-11}$ cm^2/s at 32 °C, and Fahey and Webb[27] reported $D \approx 4 \times 10^{-11}$ cm^2/s at 25 °C. In other FPR studies on DPL, Fahey and Webb[27] reported a D of $\approx 1.4 \times 10^{-10}$ cm^2/s in large vesicles near 38 °C. Their work also indicates that D in single bilayers (black lipid membranes) is faster than in oriented multibilayers and vesicles but is preparation dependent and is not affected by the phase transition.[27]

We consider our present results on DPL liposomes at less than full hydration to be in general agreement with the FPR findings. From the standpoint of the physical state of the lipids, the best comparison is probably with the large vesicles; our value at 25 °C is numerically identical with the FPR results at 38 °C[27] but there is insufficient data to correct for the temperature differences and hydration. In part because of the paucity of data on these systems, it is impossible to determine at this time whether the order of magnitude difference between our results and the FPR data on oriented multibilayers is significant. A potential contributor to the difference is that most of the instrumental instability errors in pulsed NMR measurements of D tend to give an effective D which is larger[5b] than the actual D; we have no evidence that such errors are present. We should, however, point out that it is perhaps significant that our technique and the triplet kinetics technique, both of which tend to give D faster than the FPR data, differ in one fundamental aspect from FPR: the time scale of the measurement. Our work measures motion for a period of roughly 50 ms and the kinetics method uses an even shorter time scale; during such intervals the random walk root-mean-square displacement of the lipids below T_c is at most about 100 Å. The FPR experiments, however, monitor motion for periods of several seconds to several hundred seconds, intervals in which the lipids are displaced by distances of microns. The difference in average displacement may be responsible for a different average D. Such a speculation has been offered[5b] for differences between FPR[14] and oriented sample NMR measurements of D in DPL/cholesterol systems above T_c.

In summary, we feel that this technique can quite usefully study diffusion as slow as 10^{-11} cm^2/s. The results for DPL are of the order of those obtained by other methods. Our technique has the advantage over the optical and ESR methods in that it does not require a bulky probe moiety and it has the enormous advantage over most other NMR methods in that it can be applied to rigid media. Diffusion studies in phospholipids below the phase transition have important applications in immunochemistry,[3] in particular in relating hapten mobility to the degree of complement depletion, and further applications of this technique can provide significant information in that area.

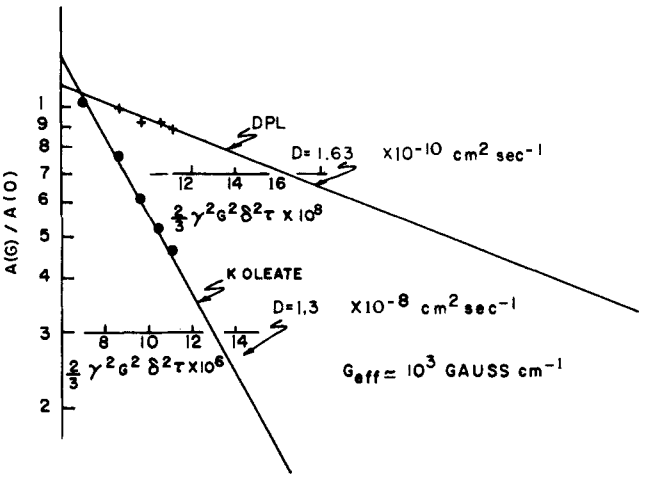

Figure 3. Experimental results for DPL and potassium oleate. Top curve DPL. Bottom, potassium oleate. The plots exhibit ln $(A(G)/A(0))$ vs. $(2/3)\gamma^2G^2\delta^2$. Note that the scales of the abscissas differ by a factor of 10^2 for the two plots shown.

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Thermochemistry and Unimolecular Reactions of Ionized Acetic Acid and Its Enol in the Gas Phase

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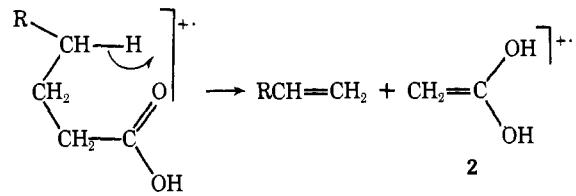
Abstract: The heats of formation of the molecular ion of acetic acid (**1**) and its tautomer $[CH_2{=}C(OH)_2]^{+\cdot}$ (**2**) have been obtained from ionization and appearance energies measured with energy selected electrons. The enolic ion, **2**, has $\Delta H_f = 120 \pm 1$ kcal mol^{-1} and is 22 ± 2 kcal mol^{-1} more stable than its ketonic tautomer, **1**. A detailed examination of metastable peak shapes for the losses of OH· and H_2O from **1** and **2** and appropriate deuterium-labeled analogues led to the following conclusions. The loss of OH· from **1** takes place at the thermochemical threshold for production of $[CH_3CO]^+$, but OH· loss from the enolic ion, **2**, takes place at an appreciably higher energy. This excess energy may be ascribed to that required for a concerted 1,3 hydrogen shift, again to yield the acetyl cation. Some interconversion of **1** and **2** does take place among ions having sufficient energy to fragment in the microsecond time frame but this process is slow relative to the fragmentation reactions. Loss of H_2O from **1** must yield $[CH_2CO]^{+\cdot}$ as daughter ion but at an energy ca. 0.6 eV (15 kcal mol^{-1}) above the thermochemical threshold. Loss of H_2O from **2** requires a larger energy (possibly ca. 1.2 eV) but again generating the ketene molecular ion. The chief significance of these results is to show the importance of keto and enol forms of ionized acetic acid as reacting configurations for fragmentation reactions and that the two tautomers are not in equilibrium in these gas-phase systems.

Introduction

The thermochemistry and gas-phase unimolecular reactions of the simple tautomeric molecular ions, acetaldehyde and vinyl alcohol, have recently been described.[2] It was found that the ionized enol was 15 kcal mol^{-1} more stable than the acetaldehyde molecular ion ($\Delta H_f = 196$ kcal mol^{-1}) and that the two ions could be easily identified from the shapes of the metastable peaks associated with the loss of a hydrogen atom.

The present work continues the examination of keto–enol cations and describes observations made on the molecular ion of acetic acid and its enolic form.

Previous experiments have shown that ionized acetic acid (**1**) generates important metastable peaks in its mass spectrum, corresponding to loss of OH· and H_2O.[3-5] Similar metastable peaks appear in the mass spectrum of the enol[3-5] (**2**) which can



2

unequivocally be generated by loss of an olefin from ionized butanoic acid (or a homologue) via a McLafferty rearrangement.[6]

The heat of formation, ΔH_f, of **1**, 142 kcal mol^{-1}, is well established (see Table I). ΔH_f(**2**) has recently been estimated to be 144 kcal mol^{-1},[5] making the keto form apparently more stable than its tautomer.

Activation energies for the losses of OH· and H_2O from **1** and **2** have also been estimated[4,5] and a lower limit of 195 kcal mol^{-1} has been set for the interconversion of **1** and **2**. The metastable peak abundance ratios for losses of hydroxyl and water from the unlabeled[3] and deuterium-labeled tautomers[4,5] have been studied and it was concluded[5] that **2** undergoes a rate-determining 1,3-hydrogen shift to yield **1** prior to loss of OH·. It was also concluded that **1** and **2** eliminate H_2O *without* interconversion but yielding ionized ketene in each case.[5] Collisional activation (CA) mass spectra have been used[4] to show that **2** ions of low internal energy do not readily isomerize to **1**, and that ionized ketene is produced by loss of H_2O from both tautomers.[5]

The purpose of the present investigation was firmly to establish the heat of formation of the enol **2**, to measure the activation energies for the losses of OH· and H_2O from **1** and **2**, and to examine in detail the shapes of the corresponding metastable peaks. It was hoped that the metastable peak shapes would unequivocally show whether **1** and **2** fragment independently by OH loss or whether the rate-determining isomerization referred to above governed the metastable fragmentations of **2**.

Results and Discussion

Ionic Heats of Formation. The electron impact ionization

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