Determination by Proton Nuclear Magnetic Resonance of the Enol, Hydrate, and Keto Forms of Oxaloacetic Acid and Its Anions

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NMR spectroscopy has been used to determine the concentrations of the hydrate, keto, and enol forms of oxaloacetic acid and its dianion in aqueous solution at 38 °C. At this temperature the spectrum of the monoanion displays peaks for only the keto and hydrate forms, but at 4 °C the enol signal also can be seen. The existence of the hydrate form of oxaloacetic acid and its dianion had been inferred from a study of the malate dehydrogenase catalyzed reduction of oxaloacetate dianion, but this is the first proof of its existence. Our results are compared with earlier NMR and spectroscopic studies where only the keto and enol forms were considered. The significance of hydration of oxaloacetic acid with respect to the kinetics of decarboxylation is discussed. The hydration of oxaloacetic acid in 50% Me_2SO–water and the extent of hemiketal formation in methanol have also been determined.

Because of interest in certain kinetic studies we wanted to know the extent to which oxaloacetic acid ($OAAH_2$) and its mono- and dianion ($OAAH^-$ and OAA^{2-}, respectively) are present in aqueous solution in the hydrate (*gem*-diol) form. There have been numerous determinations of the enol content by bromine titration,[1,2] uv,[3-7] and NMR[8-12] methods, but none of these studies have discussed a hydrate form. However, Pogson and Wolfe[13] have observed three relaxation times in the malate dehydrogenase catalyzed reduction of OAA^{2-} by NADH. They attribute the slower two of these to the ketonization of the enol and hydrate forms of OAA^{2-}, and report that the hydrate is present to the extent of 7.8%. By similarly reducing a rapidly neutralized solution of $OAAH_2$ they report a hydrate content of 55% for the diacid.

We have examined the NMR spectra of oxaloacetic acid and its anions in H_2O and identified and determined the intensities of peaks due to the enol, keto, and hydrate forms.[29] These results are compared to those from earlier studies, and discussed with respect to the kinetics of decarboxylation of oxaloacetic acid and its anions. We have also observed that for the conditions used in these experiments the signal for the enol form of the monoanion is too broad to observe at 38 °C, possibly because of concerted general acid–general base catalysis of the enol $\rightleftarrows$ keto equilibration.

Experimental Section

Reagents. *cis*-Oxaloacetic acid, grade I, 90–95%, mp 150–154 °C dec, was purchased from Sigma and used as received. As noted in the Results section, this material contained a small quantity of acetic acid, but no other impurities were detected by NMR. 2,2-Difluorosuccinic acid and sodium pyruvate, type II, dimer free, also were purchased from Sigma, sodium fluoropyruvate, A grade, was purchased from Calbiochem, and pyruvic acid was Eastman "highest purity"; all were used without further purification. Carbonate-free KOH solutions were prepared using J. T. Baker "Dilut-It" concentrates. Dimethyl sulfoxide-d_6 (Me_2So-d_6), 99.5% D, D_2O, 99.8% D, and methanol-d_4 were purchased from Stohler Isotope Chemicals. An NMR spectrum of the neat Me_2SO-d_6 showed a small –OH signal (confirmed by adding H_2O) and thus this solvent was not totally dry. Methyl-d_3 alcohol (CD_3OH), 99% D, was obtained from Merck Sharp & Dohme Canada. Absolute methanol was J. T. Baker "spectrophotometric" grade. Diethyl ether was taken from a freshly opened container and used without further purification or drying. Fluorofumaric acid, mp 232–238 °C, was prepared from 2,2-difluorosuccinic acid by the method of Raasch et al.[14] Water used was deionized, then redistilled in an all-glass still.

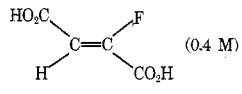
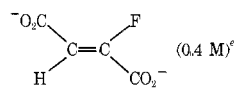
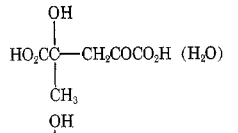
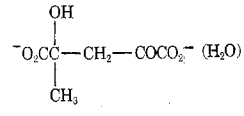
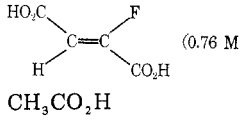
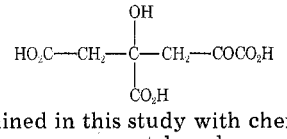
Solutions of OAA^{2-}, oxaloacetate dianion, were prepared by adding solid oxaloacetic acid to a well-stirred cold KOH solution, then back-titrating with 6 M HCl (usually only a few microliters necessary) to pH 7 using a pH meter. This procedure was intended to minimize the concentration of $OAAH^-$, oxaloacetate monoanion, which decarboxylates more rapidly than $OAAH_2$ or OAA^{2-}. In some cases the solutions also contained 0.01 M phosphate buffer. In preparing 0.4 M solutions of $OAAH_2$ or OAA^{2-}, dissolution was sufficiently rapid that the solutions could be kept cold. But for higher concentrations the solutions were allowed to warm to room temperature, then rechilled. Solutions with varying fractions of $OAAH^-$ present were prepared either by neutralizing $OAAH_2$ solutions with 1 M KOH or by reacidifying OAA^{2-} solutions with 0.1 M HCl, except that solutions of $OAAH^-$ used for peak intensity measurements were prepared by rapid mixing of equal volumes of $OAAH_2$ and OAA^{2-} (pH ~7, with no buffer) solutions using graduated pipets or a (single) Sigma Eppendorf-type pipet. pH measurements were made using a Radiometer Model 26 pH meter and a Radiometer GK 2321C combination pH electrode that was calibrated using Fisher standard buffers.

NMR spectra were obtained on a Varian A-60 NMR spectrometer, in most cases with samples at the normal probe temperature of 38 °C. Probe temperature was measured using a thermometer that could be inserted into the probe in place of the sample tube. In some of the aqueous samples DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) was included as an internal chemical shift standard. In others peak positions were measured relative to the Me_4Si signal of a separate reference sample. This latter procedure, of course, gives more approximate peak positions. In Me_2SO-d_6 solutions chemical shifts were measured with respect to internal Me_4Si. Peak intensities were measured by integrating with a planimeter the peaks in spectra obtained with a sweep width of 100 Hz and with an amplitude setting just small enough to keep the largest peak on scale. Figure 1 is included to show signal:noise under these conditions for the smaller peaks. Sweep time and filter band width settings were chosen to obtain symmetrical line shapes. We checked to see that the shape and size of each peak was unaffected by reduction in rf power or further reduction of the filter band width. For small peaks three separate tracings were obtained, and each tracing was integrated at least five times. Approximate peak widths at half height, where given, were measured on 100-Hz sweep width spectra following shimming on a sample peak. Uv measurements were made with a Unicam SP 1800 spectrophotometer equipped with a thermostatable cell holder.

Results

The NMR spectrum of 0.38 M $OAAH_2$, undissociated oxaloacetic acid, has two major peaks (singlets) at δ 3.87 and 2.97 that we assign to the keto and hydrate forms of the acid, respectively. As described below, with 1.14 M solutions a third peak assigned to the enol form can be seen 2.08 ppm downfield from the keto peak. These assignments are consistent with the spectra of a variety of model compounds; see Table I. In particular the separation of the keto and hydrate peaks is the same as observed for pyruvic acid,[15-17] but relative to pyruvic acid both peaks are shifted downfield, as would be expected as a result of replacing an H– (in pyruvic acid) by an HO_2C– (in oxaloacetic acid). Furthermore, the positions of the keto and enol peaks are nearly the same as those in diethyl oxaloacetate,[8] and the position of the hydrate peak is in agreement with that in the difluorosuccinic acid model. The spectra obtained in Me_2SO-d_6 and 50% Me_2SO-d_6–H_2O also substantiate these assignments. As the pH is increased to 7, and the diacid is converted to the mono- and dianion, the keto and hydrate peaks shift upfield by 0.27 and 0.34 ppm, respectively,

Table I. NMR Data for Model Compounds at 38 °C

Compd	Peak assignments
	D_2O Solutions
CH_3COCO_2H (0.5 M)	δ 2.44 (s) keto form δ 1.57 (s) hydrate form } keto ≃ 42%[b]
$CH_3COCO_2^-$ (0.55 M)	δ 2.33 (s) keto form δ 1.48 (s) hydrate form } keto ≃ 93%[b] (also, small peak at δ 3.35)
FCH_2COCO_2H (0.22 M)[c] $FCH_2COCO_2^-$ (0.22 M)	δ 4.53 (d) J_{HF} = 47 Hz hydrate form δ 5.47 (d) J_{HF} = 47 Hz keto form δ 4.43 (d) J_{HF} = 47 Hz hydrate form } keto ≃ 15%[b,d]
$HO_2CCH_2CF_2CO_2H$ (0.4 M)	δ 3.33 (t) J_{HF} = 15 Hz[d]
 (0.4 M)	δ 6.38 (d) J_{HF} = 30.5 Hz[d]
 (0.4 M)[e]	δ 6.17 (d) J_{HF} = 34 Hz[f]
$EtO_2CCH_2COCO_2Et$ (neat)	δ 3.84 (s) keto form δ 5.90 (s) enol form } keto = 21%[g]
	δ 1.49 (s) CH_3-[h] δ 3.25 (d) —CH_2—
	δ 1.32 (s) CH_3-[h] δ 3.27 (d) —CH_2—
	Me_2SO-d_6 Solutions
CH_3COCO_2H (~1 M)	δ 2.33 (s) keto (major peak) (plus small peaks at δ 1.58 and 6.27)
$HO_2CCH_2CF_2CO_2H$ (0.65 M)	δ 3.26 (t) J_{HF} = 15 Hz
 (0.76 M in Me_2SO-h_6)	δ 6.12 (d) J_{HF} = 31 Hz
CH_3CO_2H	δ 1.92
	δ 3.16 (s) —CH_2— next to C=O[i] δ 2.95 (s) other —CH_2— δ 6.35 (s) "product of dehydration"

[a] Determined in this study with chemical shifts relative to separate Me_4Si reference sample unless noted otherwise. [b] Approximate measurement based on peak heights. [c] Prepared from solution of sodium salt by adjustment to pH 1 with 6 *N* HCl. [d] Spectrum unchanged after 1 day. [e] Solution in NaOH, final pH 11–12. [f] Spectrum unchanged after 2 weeks at room temperature. [g] Data of Kumler et al., ref 8. [h] Data of Leussing and Stanfield, ref 15. Position of peaks converted to Me_4Si scale using chemical shift of pyruvate as common point. [i] Data of Wiley and Kim, ref 19. It seems likely that the peak at δ 6.35 is actually due to the enol form of citroylformic acid.

and the intensity of the keto peak increases relative to that for the hydrate (see Table II). The enol signal also shifts upfield by 0.37 ppm. The increase of the keto relative to the hydrate form with dissociation of $OAAH_2$ is consistent with the increased keto content of pyruvate compared to undissociated pyruvic acid.[15-17] As the pH is increased further the hydrate and keto peaks broaden. Spectra of 0.4 M solutions of $OAAD_2$ or OAA^{2-} in D_2O obtained within 5 min of mixing display only a residual –OH peak due to HDO. With 1.14 M solutions of $OAAH_2$ or with 1.14 or 1.70 M solutions of OAA^{2-} peaks for the enol as well as the keto and hydrate forms can be seen. The results of the integrations of these more concentrated solutions are listed in Table III. For the OAA^{2-} the relative amounts of the three forms is seen to be independent of the total oxaloacetate concentrations. Hence the possibility that the low-field peak is an artifact of dimer formation is unlikely.

The NMR spectrum of a 1.14 M solution of $OAAH^-$ at 38 °C shows peaks for the keto and hydrate forms, but none for the enol. However, at 4 °C a broad peak ($W_{1/2} \sim 2$ Hz) can be seen 2.00 ppm downfield of the keto peak. As the solution is warmed to 12 °C then 30 °C, the enol peak broadens and disappears and the keto peak broadens slightly while a water side band remains unchanged. Hence for the monoanion the enol ⇄ keto interconversion is sufficiently rapid at 38 °C that the enol peak is too broad to observe, even though it is observable at lower or high pH. This phenomenon is being studied further as a possible example of concerted general acid–general base catalysis. Because of rapid decarboxylation of the monoanion at 38 °C, only an approximate keto/hydrate ratio has been obtained.

The spectrum of 0.50 M $OAAH_2$ in methyl-d_3 alcohol, CD_3OH, has peaks assignable to the enol, keto, and hemiketal forms of $OAAH_2$ as well as those due to –OH and the

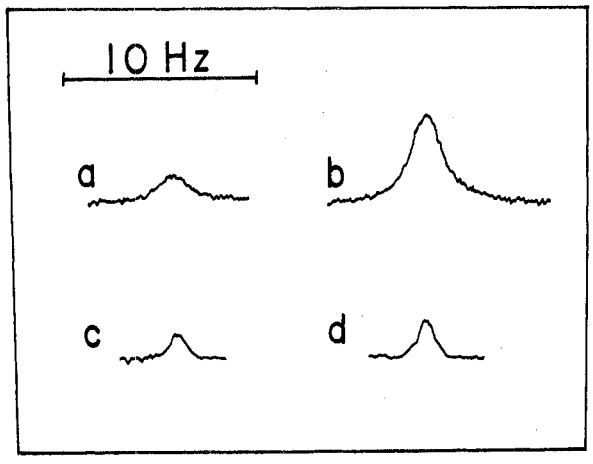


Figure 1. Examples of the signal:noise for the minor peaks in the NMR spectra of 1.14 M $OAAH_2$ or OAA^{2-} at 38 °C: (a) enol and (b) keto peaks of $OAAH_2$; (c) enol and (d) hydrate peaks of OAA^{2-}. For the $OAAH_2$ signal the spectrum amplitude is 12.5; for OAA^{2-} it is 10.0.

Table II. NMR Data for 0.38 M Oxaloacetic Acid Solutions at Various pH's at 38 °C

pH	δ (keto form)[a]	δ (hydrate form)[a]
A. 0.38 M $OAAH_2$ Solution Neutralized with KOH		
1.60	3.87	2.97
2.55	3.82	2.95
3.46	3.73	2.87
4.37	3.67	2.78
7.0	3.65	2.63
12.6	3.60 ($W_{1/2}$ ~ 6 Hz)	
B. 0.38 M OAA^{2-} (with 0.04 M Pi Buffer) Acidified with 0.1 M HCl		
10.85	3.70	
7.02	3.68	2.68
6.52	3.68	2.68
6.00	3.68	2.68
5.42	3.70	2.72
4.89	3.72	2.80

[a] Relative to separate Me_4Si reference sample.

CHD_2–group of the solvent. The methylene hydrogens of the hemiketal give rise to an AB pattern due to their nonequivalence.[18] The results of integrations of the various peaks are given in Table IV. The addition of a small amount of CH_3OH (~0.5% of total methanol) gives rise to a strong singlet. The spectrum of $OAAH_2$ in CH_3OH is much more complex due to strong ^{13}C satellite peaks and spinning side bands of the CH_3 signal, but a sharp spike ~0.1 ppm upfield of the main peak is presumably the CH_3 of the hemiketal.

Because of certain claims[2,5] that the enol form of $OAAH_2$ in methanol and ethanol is stable at ≤−10 °C, we have also looked at low temperature spectra of $OAAH_2$ in CD_3OH. The spectrum of a 0.38 M $OAAH_2$ solution that was prepared at room temperature and then cooled to −23 °C again has peaks due to enol and hemiketal, but the keto signal is no greater than the noise (for the conditions used). When we determined at −23 °C the spectrum of a second 0.38 M solution that had been prepared at −23 °C, we could initially see a strong enol and a weak hemiketal signal. After 60 min the signal due to the hemiketal was about 50% as high as when the sample was at 38 °C. The formation of the hemiketal is therefore fairly rapid even at −23 °C.

According to our NMR measurements, at 38 °C there is 4.8 times as much enol in methanol solutions of $OAAH_2$ as in aqueous solutions. But according to Hess and Reed[9] the apparent extinction coefficient at 260 nm of oxaloacetic acid in methanol is only 2.5 times as large as that in water (at 25 °C?). Therefore, in order to obtain an additional measure of the enol content of $OAAH_2$ in methanol and water we have determined at 38 °C the apparent extinction coefficients at 260 nm of oxaloacetic acid in these solvents. A 50-μl room temperature aliquot of a 8.64 mM solution of oxaloacetic acid in methanol was added to 3.0-ml samples of methanol or water that were thermostated at 38 °C. As soon as a steady absorbance reading was obtained (within 1 min), a drop of concentrated HCl was added and the absorbance remeasured. We found that in methanol ϵ_{app} = 2.19 mM^{-1} cm^{-1}, and in methanol + HCl it is 2.24 mM^{-1} cm^{-1}. In water ϵ_{app} = 0.83 mM^{-1} cm^{-1} and in aqueous HCl it is 0.49 mM^{-1} cm^{-1}. Therefore, according to these measurements, and in good agreement with our NMR results for more concentrated solutions, undissociated oxaloacetic acid is 4.6 times more enolized in methanol than in water.

We have briefly considered whether $OAAH_2$ becomes esterified in methanol under the conditions of the NMR experiments. We prepared a 0.25 M solution of oxaloacetic acid in CH_3OH and incubated it at 38 °C for 30 min, then removed the solvent with a rotary evaporator. When we directly redissolved the residue in CD_3OH, or when we first washed it by redissolving it in dry ice cooled CD_3OD, reevaporating the cold solution, and then dissolved the residue in CD_3OH, the NMR spectra of the final solutions indicated CH_3 groups, with the methyl peak about twice as high as that due to keto $OAAH_2$. Thus CH_3 groups are being carried along, presumably as either methyl ester or hemiketal groups. The figures reported for $OAAH_2$ in methanol thus may be an average of those for the free acid and its esters.

We have also examined the spectra of 0.76 M $OAAH_2$ in Me_2SO-d_6 and in 50% (by volume) Me_2SO-d_6–H_2O. In Me_2SO-d_6 peaks for the enol and keto forms are seen at 5.82 and 3.75 ppm downfield from Me_4Si with areas corresponding to 56.5% enol form. A broad low field peak due to –OH is also seen. A rescan of the spectrum within 2 min after addition of a drop or two of D_2O shows that both the enol and keto signals have decreased to a level that is the same in later rescans. If H_2O is added the –OH signal moves upfield, and a new peak at δ 2.65 assignable to the hydrate form starts to grow. In 50% Me_2SO-d_6–H_2O peaks due to the enol, keto, and hydrate forms can be seen (Table IV).

Because of claims that $OAAH_2$ is completely enolized in diethyl ether, we attempted to determine the enol content by NMR. In the spectrum of a ~0.07 M solution of $OAAH_2$ in ether we could easily detect the enol signal at δ 5.94 (relative to internal Me_4Si). The region where the keto signal is expected is just downfield of the quartet due to the methylene group of the solvent. But with varying spinning rates this region can still be examined fairly well, and we thus could see a peak 133 Hz upfield of the enol signal. In the various solvents that we have used the signals for the enol and keto forms are separated by 115 (OAA^{2-} in H_2O) to 128 Hz ($OAAH_2$ in CD_3OH), and they are separated by 129 Hz in dioxane-d_6.[9] If it is the peak for the keto form that we have observed, then based on relative peak heights $OAAH_2$ is about 90% enol in ether.

The above discussion is somewhat oversimplified in that in all cases decarboxylation is occurring. (For this reason all solutions were prepared immediately before their use.) The decarboxylation is sufficiently fast with the monoanion at 38 °C that accurate integrations were impossible. With the diacid and dianion forms very little decarboxylation occurred in the course of obtaining the NMR spectra. As noted by Kumler et al., several new peaks grow as those of oxaloacetic acid decrease. Wiley and Kim[19] have shown that at pH 3–7 citroyl-

Table III. NMR Data for Oxaloacetic Acid and Its Anions in H_2O at 38 °C

Peak assignment[a]	$W_{1/2}$, Hz[b]	Content[c]	Registry no.
A. 1.14 M $OAAH_2$ (pH 1.30)[d,e]			
δ 3.01 hydrate form	1.0	80.5 ± 1.0%	60047-52-1
δ 3.94 keto form	1.8	13.3 ± 1.3%	328-42-7
δ 6.02 enol form	1.8	6.2 ± 0.3%	7619-04-7
B. 1.14 M $OAAH^-$ [1:1 Mixture of 1.14 M $OAAH_2$ and OAA^{2-} (pH 6.89)][f]			
δ 2.91 hydrate form	0.9	keto/hydrate ~2.4	60047-53-2
δ 3.76 keto form	1.8		27531-88-0
C. 1.14 M OAA^{2-} (pH 6.89)[g]			
δ 2.65–2.70 hydrate form	0.9	5.2 ± 0.09%	60047-54-3
δ 3.64–3.70 keto form	0.6–0.7	87.4 ± 0.05%	149-63-3
δ 5.57–5.62 enol form	0.7	7.4 ± 0.13%	7314-09-2
D. 1.70 M OAA^{2-} (pH 6.70)			
δ 2.73 hydrate form	0.8	5.5 ± 0.40%	
δ 3.72 keto form	0.6	87.3 ± 0.25%	
δ 5.63 enol form	0.9	7.2 ± 0.15%	

[a] Peak positions relative to internal DSS or a separate Me_4Si sample. [b] Width at half height. [c] Average of results for two separate samples. [d] The $OAAH_2$ is <5% dissociated to monoanion under these conditions. [e] Little change in spectrum after about 1 h at 38 °C. New peaks at δ 2.98 and δ 3.25 (citroylformic acid) are seen, however. [f] Peaks at δ 2.35 (pyruvate) and 2.83 and 3.24 (citroylformate) grow as those of OAAH decrease. Did not look above 2.0 ppm. [g] Peaks at δ 2.37–2.42 (pyruvate), 2.56–2.60 (an unresolved doublet), and 3.27–3.33 (citroylformate) grow. Small peaks at δ 2.78 and 2.93 are observable initially but disappear in the course of taking expanded scale spectra.

Table IV. NMR Data for Oxaloacetic Acid in Me_2SO-d_6, 50% (by Volume) Me_2SO-d_6–H_2O, and CD_3OH at 38 °C

Peak assignment[a]	$W_{1/2}$, Hz	Content[b]
A. 0.76 M $OAAH_2$ in Me_2SO-d_6[e]		
δ 3.75 keto form[c]		43.5 ± 1.8%
δ 5.82 enol form[c]		56.5 ± 1.8%
δ 11.4–OH		
B. 0.76 M $OAAH_2$ in 50% (by Volume) Me_2SO-d_6–H_2O[f]		
δ 2.80 hydrate form	1.2	65.5 ± 0.2%
δ 3.73 keto form	1.7	18.6 ± 0.2%
δ 5.83 enol form	2.0	15.9 ± 0.4%
C. 0.50 M $OAAH_2$ in CD_3OH[g]		
δ 2.89[d] hemiketal form		60.5 ± 2.0%
δ 3.79 keto form		10.0 ± 0.3%
δ 5.93 enol form		29.5 ± 1.6%

[a] Peak positions relative to internal Me_4Si for Me_2SO-d_6 and CD_3OH solutions, or to central peak of Me_2SO-d_6 multiplet (δ 2.52) for Me_2SO-d_6–H_2O solutions. [b] Error limits represent standard deviations of multiple integrations. [c] Hess and Reed[9] report δ 3.82 (keto form) and 5.90 (enol form). [d] An AB multiplet with $\delta_{AB} = 10.2$ and $J_{AB} = 16$ Hz. [e] Peak at δ 2.34 (pyruvic acid) grows as those due to the keto and enol forms of $OAAH_2$ decrease. Peak at δ 1.92 (acetic acid) is present originally and remains apparently unchanged. Peaks at δ 4.67 and 4.98 (~equal size) are present originally but disappear. [f] Peaks at δ 2.30 and 1.39 (the keto and hydrate forms of pyruvic acid) grow as those of $OAAH_2$ decrease. Also small peaks at δ 2.75 and 2.93 (citroylformic acid) grow slowly. [g] No change in spectrum after 1 h at 38 °C.

formic acid (the product of the aldol condensation of pyruvic and oxaloacetic acids) is obtained. We will briefly discuss our observations in this regard; cf. Tables III and IV. In the case of the decarboxylation of $OAAH_2$ in Me_2SO-d_6 a peak at the position of ketopyruvic acid increases as those for oxaloacetic disappear. In 50% Me_2SO-d_6–H_2O peaks increase at δ 2.30 and 1.39 (assigned to the keto and hydrate forms of pyruvic acid) and two peaks at δ 2.75 and 2.93 (probably due to citroylformic acid) also grow more slowly. In H_2O solutions of $OAAH_2$, peaks at δ 2.98 and 3.25 (probably citroylformic acid) grow in the course of obtaining the NMR spectra, but signals due to pyruvic acid are not observed. For solutions of $OAAH^-$ or OAA^{2-} peaks assigned to pyruvate and citroylformate grow. In methanol the spectrum of $OAAH_2$ was unchanged after 1 h at 38 °C.

Discussion

Although the enol content of oxaloacetic acid and its anions has been the subject of numerous studies over a period of more than 60 years, the first and only reference to hydrate content is that of Pogson and Wolfe,[13] who studied by stopped-flow spectrophotometry the malate dehydrogenase catalyzed reduction of OAA^{2-} by NADH. Three relaxation times for this process were observed that in order of fastest to slowest rate were attributed to reduction of the keto form, dehydration of the hydrate to the keto form, and ketonization of the enol form. Based on the magnitudes of the absorbance change during each phase of the reduction it was concluded that for 26 μM OAA^{2-} at 20 ± 2 °C and pH 7.4 the hydrate, keto, and enol forms are present to the extent of 7.8, 74.3, and 17.8%, respectively. By similarly studying the reduction following a rapid neutralization of a pH 2 $OAAH_2$ solution, they concluded that for the diacid the various forms are present to the extent of 54.9, 11.1, and 34.0, respectively. Our results confirm the existence of the hydrate form, and quantitatively are in fair agreement with theirs for OAA^{2-}. But for the diacid form their results indicate significantly smaller hydrate and larger enol contents than obtained by us on more concentrated solutions.

Kumler, Kun, and Schoolery[8] reported the first study by NMR of oxaloacetic acid and its diethyl ester. The comparison of their results (summarized in Table V) to our own is complicated because they made certain assignments that we believe to be incorrect. The peak positions for the neat diester are approximately the same as those that we have reported for the enol and keto forms of the diacid in water and in Me_2SO-d_6, and are also similar to peak positions for the diacid in deuterated dioxane, Me_2SO, and acetone as reported by Hess and Reed.[9] But the peak that Kumler et al. assigned to

Table V. NMR Peak Assignments for Oxaloacetic Acid and Its Diethyl Ester According to Kumler et al.[5,a]

Compd	Solvent	δ (keto)	δ (enol)	% enol[b]
$EtO_2CCH_2COCO_2Et$	Heat	3.84	5.90	79
$EtO_2CCH_2COCO_2Et$	CH_3OH	3.23	5.91	50
$HO_2CCH_2COCO_2H$	CH_3OH	3.27	5.94	21
$HO_2CCH_2COCO_2H$	40% CH_3OH–H_2O	[c]	5.98	16
$HO_2CCH_2COCO_2H$	H_2O	[c]	6.13	8

[a] Data obtained at 29.5–33 °C. Concentrations unstated. Except for H_2O solutions, chemical shift standard was internal Me_4Si. [b] Percent enol calculated from relative peak areas. [c] Position of peak used for keto form is not given.

the keto form of the diacid or diester in methanol is about 0.6 ppm further upfield than that for the neat diester. This is just upfield of the solvent CH_3 peak, and is probably actually the CH_3 of a hemiketal derivative. Consistent with this interpretation, in the spectrum of $OAAH_2$ in methyl-d_3 alcohol we can see a signal for the methylene hydrogens of this adduct, and a signal for the keto form in the position expected, about 2 ppm upfield of the enol. The earlier assignments errors are probably a result of the fact that the keto and hemiketal methylene group signals are approximately symmetrically placed about the peak due to the CH_3 group of methanol and hence with the proper sample spinning rate are both obscured by spinning side bands. Kumler's number for percent enol in methanol is thus based on a comparison of the –CH= signal of the enol to the CH_3 of the hemiketal, and actually corresponds to an enol:hemiketal ratio of 0.40 for $OAAH_2$ (our result 0.49) and 1.5 for the diethyl ester. For $OAAH_2$ in water the strongest peak (aside from –OH) is the hydrate CH_2 at ~3.0 ppm. If we assume that this is the peak that Kumler et al. compared to the enol peak, then their figure of 8% enol (supposedly based on enol plus keto forms) is actually a percent enol based on enol plus hydrate forms. Using our figures (from Table III) of 80.5% hydrate and 6.2% enol, the percent enol calculated on Kumler's basis is 7.2%.

As mentioned above, Hess and Reed have reported NMR data for the enol and keto forms of $OAAH_2$ in deuterated dioxane, Me_2SO, and acetone. Our result of 56.5% enol in Me_2SO-d_6 is in good agreement with their figure of 53%. We did not observe, however, that decarboxylation in this solvent is a particularly rapid reaction at probe temperature, as these authors had reported.

Tsai et al.[10,11] have reported that a peak at 3.00–3.03 ppm (relative to internal DSS) in the NMR spectra of oxaloacetic acid and its two monoethyl esters can be assigned to the keto forms (and they do not report other peaks). On the basis of our work it seems likely that these peaks are actually due to hydrate forms. Since the assignments by Tsai et al. form a basis for a claim for the observation of a Eu^{3+}–keto $OAAH_2$ complex, this latter question must be reexamined with enol, keto, and hydrate forms considered, and with the effect of possible Eu^{3+}-catalyzed interchange of these forms also taken into account. Tsai[12] has also reported the determination of the enol content of the two monoethyloxaloacetic acids and their anions by NMR. These measurements were based on a comparison of the areas of the "ketonic CH_2 and esteric CH_3" peaks, but no peak positions are reported. It seems likely that the measured ratio in at least some of the cases will be a measure of the fraction of the monoester present in a hydrated form.

Spectrophotometric Determination of Enol Content. There are several reports of the use of spectrophotometry in the determination of the enol content of oxaloacetic acid and its anions that are based on the fact that near 260 nm the enol absorbs much more strongly than does the keto form. The spectrophotometric method requires that the apparent extinction coefficient [$\epsilon_{app} = A_{obsd}/(l[OAA]_{total})$, where A is absorbance and l is cell path length] be compared to the extinction coefficient of the enol, keto, and hydrate forms. The effect of ignoring the hydrate form in all studies to date is discussed below. If ϵ_{app} is the apparent extinction coefficient of an oxaloacetic acid solution and ϵ_{enol} and ϵ_{keto} are the extinction coefficients of the enol and keto forms, the hydrate being ignored, then

$$\frac{[enol]}{[enol] + [keto]} = \frac{\epsilon_{app} - \epsilon_{keto}}{\epsilon_{enol} - \epsilon_{keto}} \quad (1)$$

If the hydrate form is also explicitly considered, then eq 2 is obtained.

$$\frac{[enol]}{[enol] + [keto] + [hydrate]} = \frac{\epsilon_{app} - (\epsilon_{keto} + \epsilon_{hyd})}{\epsilon_{enol} - (\epsilon_{keto} + \epsilon_{hyd})} \quad (2)$$

Thus the fact that oxaloacetic acid and its anions are partially hydrated has no effect on the calculated fraction of enol so long as the "ϵ_{keto}" used in eq 1 is a weighted average of the extinction coefficients of the keto and hydrate forms, as it is at least for most of the studies discussed below, or so long as $\epsilon_{app} \gg \epsilon_{keto} + \epsilon_{hyd} \ll \epsilon_{enol}$, which is also usually true. Thus, even though the hydrate forms have been ignored in the spectrophotometric studies, the percent enol results are in most cases still equivalent to percent enol figures based on enol, keto, *and* hydrate forms.

The various reports of the use of this method are mainly distinguished by the way in which values of ϵ_{enol} and ϵ_{keto} were obtained. In the earliest study of this type Hantzsch[3] found that the observed extinction coefficients of $OAAH_2$ in ether and petroleum ether are the same, and nearly identical with that for the enol ether model diethyl ethoxyfumarate. Hantzsch concluded that $OAAH_2$ is completely enolized in ether, and he thus used the apparent extinction coefficient in this solvent as ϵ_{enol}. (This assumption concerning the form of $OAAH_2$ in ether was important for many of the later studies discussed below.) Using this figure and the apparent extinction of diethyl α,α-diethyloxaloacetate as a measure of ϵ_{keto}, Hantzsch concluded that in water $OAAH_2$ is 3% enol. He also noted that the monosodium and disodium salts of oxaloacetic acid absorb more strongly and thus contain more enol.

Gelles and Hay[4] confirmed that the absorption spectra of $OAAH_2$ in ether and light petroleum ether are identical and obtained $\epsilon_{app}^{260} = 8800\ M^{-1}\ cm^{-1}$, which they used as ϵ_{enol}^{260} for water solution. To obtain ϵ_{keto}^{260} they used the apparent extinction coefficients of α,α-dimethyloxaloacetic acid or its dianion since this compound cannot enolize. (But it can hydrate, and the value of ϵ_{keto} obtained in this way is a weighted average of the extinction coefficients of the keto *and* hydrate forms.) The values thus obtained, 22 and 92 $M^{-1}\ cm^{-1}$ for the diacid and dianion, respectively, can be interpreted to show that α,α-dimethyloxaloacetic acid is at least 75% hydrated. These values of ϵ_{enol} and ϵ_{keto} together with ϵ_{app} = 420 and 850 $M^{-1}\ cm^{-1}$ at 25 °C for $OAAH_2$ and OAA^{2-}, respectively, mean that $OAAH_2$ is 4.5% enol and OAA^{2-} is 8.7% enol.

Banks[2] obtained ϵ_{enol}^{280} for OAA^{2-} by extrapolating to the time of mixing the absorbance of a solution prepared by mixing an aliquot of $OAAH_2$ in ethanol (prepared and stored at −10 °C) with a pH 7.38 buffer at 1.5 °C. The value thus obtained, $\epsilon_{enol}^{280} = 3385\ M^{-1}\ cm^{-1}$, was said to be similar to the apparent extinction coefficient of $OAAH_2$ in ether, 3320 $M^{-1}\ cm^{-1}$. The apparent extinction coefficient of α-ketoglutarate measured at pH 7.38, 24 $M^{-1}\ cm^{-1}$, was used for ϵ_{keto}^{280}. For solutions of OAA^{2-} buffered at pH 5.0–10.0 at an unstated temperature Banks found that the apparent extinction coefficient is constant at 533–550 $M^{-1}\ cm^{-1}$ (except for higher values in Tris and borate buffers). She thus calculated an enol content of OAA^{2-} of 15.5–16.0%. Banks also determined an

Table VI. Summary of Spectrophotometric Determinations of the Enol Content of Oxaloacetic Acid and Its Dianion

Form	% enol			
	Reported	Calcd[a]	Calcd[b]	Ref
$OAAH_2$	4.5	4.5	3.0	4
$OAAH_2$	5.0	3.6	2.4	5
$OAAH_2$	5	5	3.3	7
OAA^{2-}	8.7	8.7	5.8	4
OAA^{2-}	15.5–15.8	10.4	6.9	2
OAA^{2-}	14.1	10	6.7	5
OAA^{2-}	15.3	10.0	6.7	6
OAA^{2-}	10	10	6.7	7

[a] Using ϵ of Gelles and Hay, ref 4. [b] Using ϵ of Hess and Reed, ref 9.

enol content of 15.5–15.8% by a bromine titration method. This is apparently for a mixture of $OAAH_2$ and its monoanion since the solutions were prepared by adding $OAAH_2$ to final concentrations of 0.24–0.38 M to 0.1 M phosphate buffer. Meyer[1] had earlier determined an enol content of 16–20% by a bromine titration method, but according to Hantzsch[3] had conceded that this figure was too high.

Tate et al.[5] followed essentially the same procedure as Banks except that $OAAH_2$ dissolved in methanol at −15 °C was added to buffers at 2 °C; from an extrapolation to the time of mixing they obtained $\epsilon_{enol}^{280} = 3607\ M^{-1}\ cm^{-1}$, which they compared to ϵ_{app}^{280} ($OAAH_2$ in ether) = $3350\ M^{-1}\ cm^{-1}$. For ϵ_{keto}^{280} they also used the apparent extinction coefficient of α-ketoglutarate dianion = $26\ M^{-1}\ cm^{-1}$. From the apparent extinction coefficient of OAA^{2-} in pH 5.2–8.4 buffers at 25 °C, which is ~$530\ M^{-1}\ cm^{-1}$, they calculated an enol content of OAA^{2-} of 14.1%. Using the same values of ϵ_{enol}^{280} and ϵ_{keto}^{280}, and ϵ_{app}^{280} ($OAAH_2$) = 205, the enol content of $OAAH_2$ was calculated to be 5%.

The various extinction coefficients obtained by Banks[2] and by Tate et al.[6] are in good agreement. However, the extinctions coefficients at 280 nm for $OAAH_2$ in ether obtained in these studies (3320–3550 $M^{supc-1}\ cm^{-1}$) are significantly lower than that reported by Gelles and Hay,[4] ~$5000\ M^{-1}\ cm^{-1}$. If Banks' and Tate's data are recalculated using this higher extinction coefficient for the enol form, then OAA^{2-} is only about 10% enol. The basis for believing that the cold alcohol solutions are pure enol form—a necessary fact if extrapolation to the time of mixing is used to obtain ϵ_{enol}—is not discussed in either study. But using bromine titrations Meyer[1] has shown that at 0 °C in absolute alcohol the enol content falls from about 100% at the time of mixing to about 50% after 3 h, and we have found that hemiketal forms in methanol solutions at −23 °C. And the fact that Tate et al. found that such a procedure when applied to the mixing of the methanolic $OAAH_2$ and HCl solutions gave an ϵ_{enol} value that was about two-thirds as large as obtained with pH 7 buffers may indicate that the absorbances changes are more complex than was believed.

Tate et al.[5] had combined the spectrophotometric measurements of enol content with potentiometrically determined pK_a's to obtain the individual acid dissociation constants of the enol and keto forms. These calculations must be reexamined in light of the presence of the additional hydrate form.

Kosicki[6] used the apparent extinction coefficients at 255 nm of $OAAH_2$ in ether to obtain $\epsilon_{enol}^{255} = 5600\ M^{-1}\ cm^{-1}$; this is somewhat smaller than the value of about $8300\ M^{-1}\ cm^{-1}$ that can be obtained from Figure 3 of the Gelles and Hay reference.[4] The apparent extinction coefficient of $OAAH_2$ in water at pH 0.5, $410\ M^{-1}\ cm^{-1}$, was used to estimate ϵ_{keto}^{255} for OAA^{2-}; for a variety of reasons this is a poorer estimate than any of those discussed so far. Kosicki found that the apparent extinction coefficient at 255 nm at pH 5–10 and 25 °C is approximately constant at $1200\ M^{-1}\ cm^{-1}$; this corresponds to 15.3% enol based on $\epsilon_{enol}^{255} = 5600\ M^{-1}\ cm^{-1}$ (or to 10.0% based on $\epsilon_{enol}^{255} = 8300\ M^{-1}\ cm^{-1}$). Without comment, Kosicki[8] later revised this figure to "10% enol in neutral solution and 5% in acid" based on an apparent extinction coefficient of $OAAH_2$ in ether of $8460\ M^{-1}\ cm^{-1}$.

Aside from the problem of the numerical value of the extinction coefficients of $OAAH_2$ in ether, there is the question of what this number represents. As discussed above, Hess and Reed[9] showed by NMR measurement that in dioxane-d_8 solution, $OAAH_2$ is only 67% enol, and therefore the true extinction coefficient of the enol at 255 nm (reported λ_{max}) is $1.1 \times 10^4\ M^{-1}\ cm^{-1}$. If the fractional enol content of $OAAH_2$ in the 0.1 mM solutions used for uv measurements is the same as in the 1.0 M solutions studied by NMR, then (since ϵ_{keto} is relatively unimportant in the calculations) ϵ_{enol} for $OAAH_2$ in dioxane is 1.5 times as large as the apparent extinction coefficient. If the same is true for diethyl ether solutions, or if ϵ_{enol} in dioxane is used to estimate ϵ_{enol} for $OAAH_2$ and OAA^{2-} in water, then all of the reported enol contents are about one-third too large. The results of the spectrophotometric studies are summarized in Table VI. If the higher extinction coefficients of $OAAH_2$ in ether are used, all of the studies agree quite closely with each other and with the NMR data.

Three groups[15–17] have used NMR to study pyruvic acid and have determined that in aqueous solution it is 54–71% hydrate while the anion is only 3–5% hydrate. In no case was the enol form detected. It is also interesting to note that whereas the substitution of a hydrogen by fluorine increases the keto content from 21% in diethyl oxaloacetate to 100% in α-fluorodiethyl oxaloacetate (measured for the neat diesters),[8] we find that in pyruvic acid or pyruvate ion this substitution leads to almost complete hydration in aqueous solution. This is in agreement with the claim based on uv absorbances that α-fluorooxaloacetic acid and its dianion are largely hydrated.[20]

Decarboxylation of Oxaloacetic Acid. Pedersen[21] found that the decarboxylation of oxaloacetic acid exhibits a bell-shaped pH dependence that can be analyzed to show that the monoanion is about 44 times as reactive as the diacid, and about 4 times as reactive as the dianion. This general picture has been confirmed by subsequent studies.[22–24] If it is assumed that only the keto forms decarboxylate, then when the difference in the keto content of oxaloacetic acid and its dianion is considered, we calculate that the keto form of the dianion is not 11 but only about 1.7 times as reactive as the keto form of the diacid. Similar considerations apply to the monoanion. Steinberger and Westheimer[25] had earlier studied the decarboxylation of α,α-dimethyloxaloacetic acid and had pointed out that the keto form of the monoanion can exist in two tautomeric forms, **1** and **2**, of which **1** should be the minor

1 **2**

but more reactive form. Consistent with this analysis they showed that the monoester anion $^-O_2CC(CH_3)_2COCO_2Et$ decarboxylates about 20 times more rapidly than the monoanion of the diacid. Gelles[22] and Tsai[24] made a similar analysis of the decarboxylation of the monoanion of oxaloacetic acid. The calculation of the reactivity of the keto form of the monoester or of the reactive tautomers of the oxaloacetate or α,α-dimethyloxaloacetate monoanions also must take into account the hydration of the keto group. Thus, if $^-O_2CCH_2COCO_2H$ is more hydrated than the dianion by the same factor that pyruvic acid is more hydrated than the pyruvate ion, then only about 30% of the proper tautomer will be

in the reactive keto form. (But in light of the proposed reactivity of tetrahedral carbinol–amine adducts in the amine-catalyzed decarboxylation[26] and enolization[27] of oxaloacetic acid, it is possible that the hydrate form can also participate in these reactions.) Leussing[28] has suggested that the formation of a dinuclear complex with hydrated α,α-dimethyl oxaloacetate can account for the inhibition of decarboxylation of this ion that is observed at high metal ion concentrations.

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References and Notes

(1) K. Meyer, *Chem. Ber.*, **45**, 2843 (1912).
(2) B. E. C. Banks, *J. Chem. Soc.*, 5043 (1961).
(3) A. Hantzsch, *Chem. Ber.*, **48**, 1407 (1915).
(4) E. Gelles and R. W. Hay, *J. Chem. Soc.*, 3673 (1958).
(5) S. S. Tate, A. K. Grzybowski, and S. P. Datta, *J. Chem. Soc.*, 1372 (1964).
(6) G. W. Kosicki, *Can. J. Chem.*, **40**, 1280 (1962).
(7) R. G. Annett and G. W. Kosicki, *J. Biol. Chem.*, **244**, 2059 (1969).
(8) W. D. Kumler, E. Kun, and J. N. Shoolery, *J. Org. Chem.*, **27**, 1165 (1962).
(9) J. L. Hess and R. E. Reed, *Arch. Biochem. Biophys.*, **153**, 226 (1972).
(10) C. Reyes-Zamora and C. S. Tsai, *Chem. Commun.*, 1047 (1971).
(11) C. S. Tsai, Y. T. Lin, C. Reyes-Zamora, and J. A. Fraser, *Bioinorg. Chem.*, **4**, 1 (1974).
(12) C. S. Tsai, Y. T. Lin, and E. E. Sharkawi, *J. Org. Chem.*, **37**, 85 (1972).
(13) C. I. Pogson and R. G. Wolfe, *Biochem. Biophys. Res. Commun.*, **46**, 1048 (1972).
(14) M. S. Raasch, R. E. Miegel, and J. E. Castle, *J. Am. Chem. Soc.*, **81**, 2678 (1959).
(15) D. Leussing and C. K. Stanfield, *J. Am. Chem. Soc.*, **86**, 2806 (1964).
(16) V. M. Becker, *Ber. Bunsenges. Phys. Chem.*, **68**, 669 (1964).
(17) V. Gold, G. Socrates, and M. R. Crampton, *J. Chem. Soc.*, 5888 (1964).
(18) Cf. A. Loewenstein and J. D. Roberts, *J. Am. Chem. Soc.*, **82**, 2705 (1960).
(19) R. H. Wiley and K.-S. Kim, *J. Org. Chem.*, **38**, 3582 (1973).
(20) E. Kun, D. R. Grasetti, D. W. Fanshier, and R. M. Featherstone, *Biochem. Pharmacol.*, **1**, 207 (1958).
(21) K. J. Pedersen, *Acta Chem. Scand.*, **6**, 285 (1952).
(22) E. Gelles, *J. Chem. Soc.*, 4736 (1956).
(23) G. W. Kosicki and S. N. Lipovac, *Can. J. Chem.*, **42**, 403 (1964).
(24) C. S. Tsai, *Can. J. Chem.*, **45**, 873 (1967). This author reports that he studied the decarborylation of oxaloacetic acid in D_2O and that of deuterated oxaloacetic acid in H_2O. In view of the rapid exchange of the carbon bound H or D with those solvents this seems very surprising.
(25) R. Steinberger and F. H. Westheimer, *J. Am. Chem. Soc.*, **73**, 429 (1951).
(26) R. W. Hay, *Aust. J. Chem.*, **18**, 337 (1965).
(27) P. K. Bruice and T. C. Bruice, *J. Am. Chem. Soc.*, **98**, 844 (1976).
(28) N. V. Raghavan and D. L. Leussing, *J. Am. Chem. Soc.*, **98**, 723 (1976).
(29) **Note Added in Proof.** An NMR study of oxalo-2-propionic acid(α-methyloxaloacetic acid) has been recently reported: N. Y. Sakkab and A. E. Martell, *J. Am. Chem. Soc.*, **98** 5285 (1976).

Sterically Stabilized Enols. A Study Employing the Internal Rotational Barriers of the Destabilized Ketones[1]

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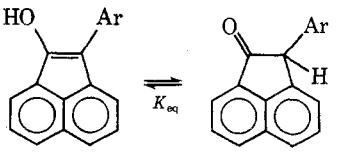
Equilibrium constants for enols 2-(2,4,6-triisopropylphenyl)acenaphthylenol (**1**) and 2-mesitylacenaphthylenol (**2**) formed from the respective sterically destabilized ketones 2-(2,4,6-triisopropylphenyl)acenaphthenone (**3**) and 2-mesitylacenaphthenone (**4**) were measured in several solvents, with maximum values of K_{eq} = 2.6 and 0.3 being observed for **1** and **2**, respectively, in Me_2SO solution. The variation of the internal rotational barrier heights as a function of the rotor's geminal substituent allows an estimate of relative ketone ground-state strain, the relaxation of which contributes the primary source of enol stability. For ketone **4** in trichlorobenzene solution, the acidity independence of the aryl site-exchange barrier and the free-energy difference between tautomers allow a determination of the lower limit of the enol's ketonization barrier as $\Delta G^{\ddagger} > 19$ kcal/mol. Enol **1**, tautomeric to the even more rotationally restricted ketone **3**, was isolated and characterized. Although the enols are of low relative free energy, a deuterium labeling experiment indicates that they are not intermediates in the pinacol rearrangement by which the respective ketones are prepared. The enols and their enolates appear useful as spectrophotometric probes of solute-solvent interactions.

Steric hindrance can selectively raise the potential energy of a keto tautomer relative to its enol form. For example, diarylacetaldehydes[3] have been sufficiently destabilized that the enols are the more thermodynamically stable tautomers. Besides increasing the energy of the keto form, steric hindrance increases the kinetic barrier to tautomerism as well; and several examples of isolable simple[4] enols of sterically hindered ketones are only kinetically stable.[5] In this category are several polyaryl enols[6] and the steroid 3β,12-dihydroxy-Δ^{12}-ursene.[7] In the present study, an examination is made of the energetics of tautomerism of sterically stabilized enols 2-tipyl-1-acenaphthylenol (**1**) (tipyl = Tip = 2,4,6-triisopropylphenyl) and 2-mesityl-1-acenaphthylenol (**2**) by studying



1, Ar = Tip
2, Ar = Mes
3, Ar = Tip
4, Ar = Mes

the barriers to internal rotation in the destabilized ketones 2-tipylacenaphthenone (**3**) and 2-mesitylacenaphthenone (**4**), respectively. As was indicated by this study, enol **1** is isolable.

Results and Discussion

The enol and destabilized keto forms are of comparable energy, the predominance of either being controlled by choice of solvent. In solvents which cannot accept hydrogen bonds, the ketones predominate, the enols existing in only trace concentrations. However, in hydrogen-bond accepting solvents, the colorless ketones give solutions which are orange, the color of the enols.[8] The equilibrium constants, K_{eq}, for the formation of the enols decrease qualitatively as a function of solvent in the order Me_2SO > DMF > EtOH > HOAc > hexane, which is the expected order of H-bond accepting ability.[9] For enol **1**, the visible absorption maximum at 440 nm allowed the K_{eq} to be estimated as 2.6 (in Me_2SO), 1.0 (DMF), 0.25 (EtOH), 0.17 (HOAc/HCO_2H), and ≤0.004 (in hexane). For enol **2**, an equilibrium constant of K_{eq} = 0.3 in Me_2SO-d_6 solution was determined by the NMR spectrum.