[CONTRIBUTION FROM THE DEPARTMENT OF PHARMACEUTICAL CHEMISTRY, SCHOOL OF PHARMACY, AND THE DEPARTMENT OF PHARMACOLOGY AND BIOCHEMISTRY, SCHOOL OF MEDICINE, THE UNIVERSITY OF CALIFORNIA, AND VARIAN ASSOCIATES]

The Enolization of Oxaloacetic Acid, Diethyl Oxaloacetate, and Diethyl Fluorooxaloacetate as Determined by NMR Analyses[1]

W. D. KUMLER, ERNEST KUN,[2] AND JAMES N. SHOOLERY

Received October 9, 1961

The per cent of enol in oxaloacetic acid was measured by NMR and found to be 8% in water, 16% in 40% water-methanol, and 21% in methanol. Diethyl oxaloacetate has 79% enol measured neat and 50% enol in methanol solution. Diethyl fluorooxaloacetate had no detectable enol when measured neat and is therefore virtually 100% keto. When pure oxaloacetic acid is placed in water solution, the NMR spectrum gives evidence of appreciable amounts of the presence of some species other than the keto and enol forms of oxaloacetic acid.

The major role of oxaloacetic (OOA) acid in intermediary metabolism justifies a careful study of available information concerning its keto-enol tautomerization equilibrium. The numerical value of this equilibrium constant and factors affecting it play a significant role in the determination of kinetic constants of enzymatic reactions of oxaloacetic acid. In addition, quantitative evaluation of the well known inhibitory effect of oxaloacetic ion,[3,4] which is presumably due to the enol tautomer,[5] also depends on the knowledge of the keto-enol ratio under defined conditions. In spite of the obvious importance of this equilibrium constant, relatively little attention has been focused on its accurate determination. This is readily explained by the experimental difficulties which hinder the unequivocal measurement of the keto-enol equilibrium. Since the publication of Kornberg,[6] the absorption band of an aqueous solution of OAA exhibited between 242 and 262 mμ was generally accepted as the absorption spectrum of this acid. This band in the ultraviolet region was extensively used for quantitative rate studies of enzymic reactions where OAA was the product of the reaction.[7,8] More recently, the enol-borate complex of OAA, which absorbs light in the same region, was employed for the same type of enzyme assay.[9] It appears to be obvious that the ultraviolet band of OAA is a measure of the enol species of unknown percentage and the actual concentration of this tautomer is subject to variation, depending on pH, and the presence of cations. Consequently, a molar extinction coefficient, representing the enol tautomer, is subject to considerable uncertainty.[10] Gelles and Hay[11] carried out spectrophotometric studies with OAA and its esters in ether and light petroleum ether and obtained ϵ 8800 at λ_{max} 260 mμ. This was assumed to be the proper extinction coefficient of the enol tautomer, as under these conditions only this species was supposed to be present. A perchloric acid solution of OAA yielded ϵ 420 at λ_{max} 260 mμ and a comparison of these values prompted these workers to conclude that OAA in its undissociated form contains 4–5% enol, while (from measurements at *p*H 6), the dianion of this acid has 9% of the enol tautomer present. These values depend on the validity of the assumption that the extinction coefficient in organic solvents and in aqueous conditions is the same, an assumption that has a high probability of not being true.

On the basis of bromine titrations, Meyer[12] stated that about 20% of OAA in aqueous solution is present as the enol, while Hantzsch,[13] again employing spectrophotometry, concluded that only 3% of the enol is present in aqueous OAA solutions. In the course of studies dealing with the mechanism of hydrogen transfer between substrates and pyridine nucleotide coenzymes and that of the decarboxylation of OAA, Vennesland[14,15] brought forward convincing evidence suggesting that the keto tautomer of OAA is the true substrate of both maleate dehydrogenase and phosphoenol pyruvate carboxylase.

(1) Supported by Grant CY-3211-C4 of the United States Public Health Service.

(2) Established investigator of the American Heart Association.

(3) A. B. Pardee, and V. R. Potter, *J. Biol. Chem.*, **176**, 1075 (1948).

(4) D. B. Tyler, *J. Biol. Chem.*, **209**, 395 (1955); *Biochem. J.*, **76**, 293 (1960).

(5) L. Hellerman, O. K. Reiss, S. S. Parmar, J. Wein, and N. L. Lesser, *J. Biol. Chem.*, **235**, 2468 (1960).

(6) A. Kornberg, S. Ochoa, and A. H. Mehler, *J. Biol. Chem.*, **174**, 159 (1948).

(7) P. P. Cohen, *J. Biol. Chem.*, **136**, 565 (1940).

(8) L. I. Feldman and I. C. Gunsalus, *ibid.*, **187**, 821 (1950).

(9) E. Kun, D. W. Fanshier, and D. R. Grassetti, *J. Biol. Chem.*, **235**, 416 (1960).

(10) M. Garzia-Hernandez and E. Kun, *Biochim. Biophys. Acta*, **24**, 78 (1957).

(11) E. Gelles and R. W. Hay, *J. Chem. Soc.*, 3673 (1957).

(12) K. H. Meyer, *Ber. deut. chem. Ges.*, **45**, 2843 (1912).

(13) A. Hantzsch, *Ber. deut. chem. Ges.*, **48**, 1407 (1915).

(14) F. A. Loewus, T. T. Chen, and Birgit Vennesland, *J. Biol. Chem.*, **212**, 787 (1955).

(15) J. L. Graves and Birgit Vennesland, *ibid.*, **223**, 551 (1956).

In an attempt to resolve some of these uncertainties, we have investigated the percentage of enol in oxaloacetic acid in NMR.

The oxaloacetic acid was from the California Foundation for Biochemical Research Company. *Anal.* Calcd. for $C_4H_4O_5$: C, 36.50; H, 3.03. Found: C, 36.53; H, 3.10. Calcd. equiv. weight, 132. Found, 132. The observed m.p. was 152°, and the literature m.p. for cis-enol oxaloacetic acid is 152°. The solutions on which NMR measurements were made were tested for the presence of pyruvic acid with 2,4-dinitrophenylhydrazine and paper chromatography and none was found. The evidence indicates that the compound used was oxaloacetic acid of better than 99% purity.

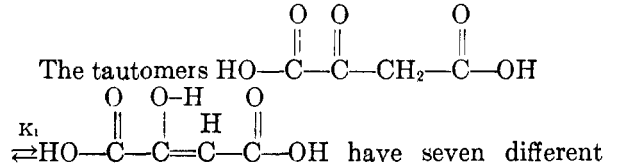
NMR spectra were obtained with a Varian HR 60 NMR spectrometer and with a Varian A 60 analytical NMR spectrometer. Both instruments operated at 60 mc./sec. in a field of 14,092 Oersteds. Tetramethylsilane was added to all samples except aqueous solutions (in which it is insoluble) to serve as an internal reference compound. Chemical shifts are reported in parts per million (p.p.m.) relative to the reference compound, according to the following definition

$$\delta(\text{p.p.m.}) = 10^6 \times (H_{ref} - H)/H_{ref}$$

Both spectrometer systems were equipped with electronic integrators for measuring the areas under the various NMR absorption peaks. These areas are linearly proportional to the number of hydrogen atoms in the chemical environment represented by the corresponding peak. The sample temperature was 33° in the HR 60 instrument and 29.5° in the A 60 instrument.

RESULTS

The tautomers



$\overset{K_1}{\rightleftarrows}$

have seven different kinds of hydrogen atoms and presumably might give rise to seven different peaks. Only three peaks would be expected, however, because the carboxyl and enol hydrogens would exchange so rapidly with themselves and with the water that only one peak would result from them and the other two peaks would come from the CH_2 and =CH hydrogens. Other peaks, however, were present in the water solution spectrum. These were not due to impurities in the original oxaloacetic acid because on the basis of all the tests which could be applied to it, such as melting point, equivalent weight, and elementary analysis, the compound was of high purity but other species were evidently formed when pure oxaloacetic acid is placed in water. The fact that the water solutions develop a yellow color after a short time is also an indication that other molecular species are present.

The per cent of keto in these solutions was estimated by measuring the CH_2 peak with the integrator and comparing this with the total hydrogen as measured in deuterium oxide. This turned out to be about 50%, but it does not follow that the rest is enol because of the presence of the other unidentified molecular species in the solutions.

The per cent of enol can be determined if the =CH peak can be identified, but it was not clear at first which this was in the water and deuterium oxide solution spectra. To find out where to expect this peak, the spectrum of diethyl oxaloacetate was measured in neat and methanol and that of oxaloacetic acid in methanol, 40% water-methanol, and water. Diethyl oxaloacetate had the peaks expected for hydrogens in the CH_3 and CH_2 in the ethyl groups, the CH_2 between the carbonyl groups, the =CH, and the OH groups. No other peaks were present indicating the compound was of high purity. The =CH peak occurred at $\delta = 5.90$ and the CH_2 (between the carbonyls) peak at $\delta = 3.84$, giving a separation of $\delta = 2.06$. The relative integrated intensity of the CH_2 peak in diethyl oxaloacetate was 59 and that of the =CH peak 110. As two hydrogens are involved in CH_2, the per cent of enol in this compound is $110/(59/2 + 110) = 79\%$.

It is of interest that the peaks for the CH_3 and CH_2 in the ethyl groups indicate that the two ethyl groups in the compound have a slightly different environment as would be expected.

The position of the =CH peak was found to be as follows: diethyl oxaloacetate, neat $\delta = 5.90$; diethyl oxaloacetate in methanol, $\delta = 5.91$; oxaloacetic acid in methanol, $\delta = 5.94$; oxaloacetic acid in 40% water-methanol, $\delta = 5.98$; oxaloacetic acid in water, $\delta = 6.13$. The CH_2 peak of diethyl oxaloacetate in methanol was at $\delta = 3.23$ and that for oxaloacetic acid in methanol was at $\delta = 3.27$, so both the =CH peak and the CH_2 peak shift approximately the same amount in going from the ester to the acid.

The per cent of enol in the compounds from this study and from the literature are given in Table I.

TABLE I

PER CENT ENOL

Compound	Solution: Water	40% Water-methanol	Methanol	Neat
Oxaloacetic acid	8 3 (13) 4–5 (11) 20 (12)	16	21	
Diethyl oxaloacetate			50	79
Diethyl fluorooxaloacetate				0

The per cent of enol in diethyl oxaloacetate neat and of oxaloacetic acid in methanol and in water was determined by using the integrator on the CH_2 and =CH peaks. The per cent of enol of diethyl oxaloacetate in methanol and the per cent of enol of oxaloacetic acid in 40% water-methanol was determined from the CH_2 and =CH peak heights and widths, as this method appeared more accurate than using the integrator because of the interference of the strong methyl signal from the solvent.

The large difference between the 79% enol in neat diethyl oxaloacetate and the 8% enol in oxaloacetic acid in water solution is in keeping with the shift towards the enol form of compounds in the pure state or in nonpolar solvents as compared with the per cent of enol in water. Acetyl acetone neat, is 80%[16] enol but only 15%[17] enol in water.

The NMR spectrum of diethyl fluorooxaloacetate which was measured neat is of interest in this connection. It contains peaks for the CH_3, CH_2 and F—C—H hydrogens only and indicates the compound is of high purity. It contains no peak for an enolic hydrogen and the peak for the hydrogen on the carbon to which fluorine is attached is split into two peaks which have a separation of 47 c.p.s. This is the amount of separation in keeping with what is expected for a spin-spin splitting resulting from a fluorine atom on the same carbon atom. The NMR spectrum therefore indicates this compound is 100% keto.

It is interesting that substitution of a fluorine for a hydrogen in diethyl acetoacetate would have such a large effect that it increased the per cent of keto from 21 to 100%. It is not without precedence, however, for introduction of a halogen to increase the per cent of keto in a compound. Introduction of a bromine into acetylacetone increases the per cent of keto from 84.5 to 91.9% when both are measured in water.

Of considerable interest is the finding that although we start out with what is quite pure oxaloacetic acid, the NMR spectrum of the water solution indicates the presence of peaks which are not accounted for by the enol and keto forms of oxaloacetic acid. When the total amount of hydrogen in oxaloacetic acid is measured in deuterium oxide using the integrator, about 80% shows up as HDO, indicating it has exchanged, while 20% is present as some other form that has not exchanged. As we used 0.1 g. of oxaloacetic acid in 1.0 g. of deuterium oxide, the number of equivalents of deuterium calculated to be present is $1.0 \times 2/20 = 0.1$ and the number of equivalents of hydrogen from the oxaloacetic acid is $0.1 \times 4/132 = 0.003$. If the exchange takes place on a purely statistical basis, then ($0.003 \times 100/0.1 = 3\%$) only 3% of the hydrogen should show up in forms other than HDO. The fact that 20% of the hydrogen has not exchanged indicates appreciable amounts of other species are present in water solutions of oxaloacetic acid and their nature is a matter for further study.

SAN FRANCISCO, CALIF.
PALO ALTO, CALIF.

(16) J. B. Conant and A. F. Thompson, Jr., *J. Am. Chem. Soc.*, 54, 4039 (1932).

(17) G. Schwarzenbach and E. Felder, *Helv. Chim. Acta*, 27, 1044 (1944).

[CONTRIBUTION FROM THE DETROIT RESEARCH LABORATORIES OF ETHYL CORPORATION AND THE DEPARTMENT OF CHEMISTRY, ISRAEL INSTITUTE OF TECHNOLOGY]

Ultraviolet Spectra of Organometallic Compounds[1,2]

RICHARD T. LUNDQUIST[3a] AND MICHAEL CAIS[3b]

Received August 15, 1961

Evidence is presented for the existence of electronic absorption bands typical of the transition metal-carbon bond. This evidence is based on a correlative qualitative study of the ultraviolet spectra of organo derivatives of iron, manganese, chromium, and molybdenum. The characteristic features of these spectra are described.

The discovery of dicyclopentadienyliron (ferrocene)[4] and the subsequent proposal[5] of the *pi*-bonded "sandwich" structure for this compound has led to the synthesis of a large number of new com-

(1) A preliminary report of this paper was presented at the Meeting of the XVIIth International Congress of Pure and Applied Chemistry, Munich, September 1959, see M. Cais and R. T. Lundquist, Abstracts of Papers, A104; and the 7th Detroit Anachem Conference, Detroit, Mich.. October 1959.

(2) "Organometallic Studies," part VI in the Israel Institute of Technology Series. Part V, M. Cais and M. Feldkimel, *Tetrahedron Letters*, 13, 444 (1961).

(3) (a) Present address: Aerojet-General Corporation, Sacramento, Calif. (b) Present address: Israel Institute of Technology, Haifa, Israel, to whom inquiries should be addressed.

(4) T. J. Kealy and P. L. Pauson, *Nature*, 168, 1039 (1951).

(5) R. B. Woodward, M. Rosenblum, and M. C. Whiting, *J. Am. Chem. Soc.*, 74, 3458 (1952).