Studies on Phenylpyruvic Acid

I. Keto-Enol Tautomerism *

O. Sciacovelli, A. Dell'Atti, A. De Giglio, and L. Cassidei

Istituto di Chimica fisica del l'Università di Bari

(Z. Naturforsch. 31 c, 5–11 [1976]; received July 1/October 6, 1975)

Phenylpyruvic Acid, Tautomerism

The tautomerism of phenylpyruvic acid (PPA) and its sodium salt was investigated. The ^1H and ^{13}C spectra of PPA in aprotic solvents and in methanol show almost complete prevalence of the enol form, whereas the keto form prevails only in water. The Z configuration was assigned to the sole enol tautomer present on the basis of the value of the vicinal coupling constant $|{}^3J_{^1H,^{13}COOH}| = 3.7$ Hz. A small amount of the hydrated form of PPA (1-phenyl-2,2-dihydroxyl-propanoic acid) was found in the aqueous solution of its sodium salt and in buffer solution (pD = 6) of PPA.

By means of infrared spectroscopy one can conclude that crystalline PPA is in the enol whereas its sodium salt is in the keto form. The keto-acid was not obtained in the solid state. The colorimetric method for testing PPA traces in urine depends on the formation of a enol-Fe^{3+} complex (2:1) which appears stable in dimethylsulfoxide (DMSO).

1. Introduction

Phenylpyruvic acid (PPA) is a product of the metabolism of phenylalanine in patients afflicted with phenylketonuria [1]. It accumulates in the blood and tissues and is excreted in the urine [2] in which it is normally detected by a colorimetric method based on a reaction with ferric chloride [3, 4].

PPA can exist in keto and enol forms [5–7]. The solid is probably the enol tautomer [6]. The keto tautomer has never been prepared as a solid. In solution, on the contrary, both forms [7] are present.

Josien *et al.* [5] presume the presence, besides the keto form, of two enol isomers in CCl_4 by means of IR studies. According to Strehlow [8] PPA should show extensive enolization in water because of the stabilization due to conjugation of the double bond with the aromatic ring and he has not found any trace of a keto-hydrated form.

Larsen and Wieczorkowska [6] find that in freshly prepared DMSO solution only the enol isomer, probably with Z configuration, is present while in 1 M HCl at equilibrium the enol percentage is 10%; they do not consider hydration.

Hence there are as yet no complete data on the states and relative concentrations of this substance in solution. Because such topics play an important role in elucidating the mechanism of the metabolic reactions in which this substrate is involved [7, 9], we have been engaged in a detailed investigation of the tautomeric equilibrium of this substance in different solvents and of its solid state with the aid of MS, ^1H and ^{13}C NMR, UV and IR spectroscopy techniques.

2. Experimental Section

Instruments and techniques

The ^1H NMR spectra were recorded at 100 MHz using a Varian HA 100 spectrometer under the experimental conditions: temperature 25 °C, expansion scale of 2 Hz mm^{-1} and sweep rate of 2 Hz s^{-1}.

Concentrations of 0.5 M were realized in organic solvents and a small amount of TMS was added to generate the internal reference and lock signal.

Because of the low solubility of PPA in water, the ^1H NMR spectrum in this solvent was obtained by using a saturated solution, and accumulating 150 scans in a Varian C-1024 time-averaging device.

H_2O was used as solvent instead of D_2O because of the slow exchange of $-CH_2-$ and $-CH=$ protons with the solvent consequent on the dynamic of the keto-enol tautomerism. In this case the solvent peak was used as lock signal by reducing the intensity of audiomodulation in the lock channel.

The solubility of PPA in buffer solution (pD = 6) was enough to give a good signal: noise ratio in a

Requests for reprints should be sent to Dr. O. Sciacovelli, Istituto di Chimica fisica dell'Università, Via Amendola 173, *Bari* (Italy).

* This work was supported financially by C.N.R. (Roma).

Abbreviations: DMSO, dimethylsulfoxide; PPA, phenylpyruvic acid.

single scanned spectrum. The same was found for the sodium phenylpyruvate in water. In both cases TSP was used as internal reference and lock sample.

The Fourier transform ^{13}C NMR spectra were measured at 25.2 MHz. The ^{13}C NMR spectra, ^{1}H noise decoupled, were recorded on a Jeol PS 100 spectrometer (1 M in DMSO-d_6, number of transients: 512, 1.25 Hz per data point). The ^{13}C NMR spectrum without ^{1}H decoupling was recorded on a Varian XL 100 spectrometer (0.5 M in DMSO-d_6, number of transients: 14 000, 0.68 Hz per data point).

The infrared spectra of crystalline PPA and its sodium salt in KBr pellets or nujol mulls were recorded on a Perkin-Elmer 257 instrument. The optical measurements were carried out using a Zeiss PMQ II spectrophotometer fitted up with a temperature-controlled cell holder. Mass spectra were obtained with a Perkin-Elmer 270 M.S. The conditions for analysis were as follows : electron-beam energy, 70 eV; ion-accelerating voltage, 2 kV; probe temperature, 60 °C; electron multiplier sensitivity, 3×10; source pressure, 1×10^{-7} mm Hg.

On studying the keto-enol equilibrium, all spectra were recorded a few hours after preparing the solutions so as to reach equilibrium.

Materials

Commercially available solvents were used. Monohydrated sodium phenylpyruvate was purchased from The British Drug Houses LTD. Poole, England. The buffer solution at pD 6 was prepared by drying an aqueous buffer solution of pH 6. The dry sample thus obtained was dissolved in D_2O, and the water sublimed out. This process was repeated three times. Then the residue was dissolved in 99.5% D_2O.

Preparation of PPA

As commercially available PPA is not very stable, it is better to obtain the acid from its more stable sodium salt every time it is required.

By the method pointed out by Berry and Woolf [4], which consists in extracting PPA with ether from a sodium salt solution in 0.1 N HCl and drying the ether solution, a product that ^{1}H NMR analysis (solvent DMSO-d_6) showed to be a mixture of phenylacetic acid (a singlet peak at $\delta = 3.7$ ppm) and PPA was obtained.

Phenylacetic acid is probably a product of oxidation of PPA, present sometimes in large quantities, due to peroxides contained in ether. Indeed the ^{1}H NMR spectrum of the product obtained by drying a solution of sodium phenylpyruvate in 0.1 N HCl shows the presence of only a minute quantity of PPA. Actually pure PPA was obtained by dissolving its salt in 0.1 N HCl, drying the solution thus prepared, washing the solid with bidistilled water to eliminate NaCl, dissolving the residue in acetone and then precipitating PPA from the solution with chloroform. PPA thus obtained melts at 155–156 °C.

3. Experimental Results

NMR spectra

The ^{1}H NMR spectra were observed for solutions of the compound in question in the following solvents with different properties: DMSO-d_6, acetone-d_6, methanol-d_4, dioxane-d_8 and water.

The spectra in deuterated dioxane and methanol show two complex multiplet patterns in the phenyl region between $\delta = 7.1$ and 7.4 ppm and between $\delta = 7.6$ and 7.8 ppm, and a singlet in the olefinic region at $\delta = 6.4$ ppm. The ratio of the integrated intensities of the signals between $\delta = 7.1$ ppm and $\delta = 7.8$ ppm to the area of the signal at $\delta = 6.4$ ppm is 5 : 1. No change of the spectra was noticed for a few hours after the preparation of the solution and in particular no trace of signal was observed in the absorption range of methylene protons.

In acetone and DMSO besides these signals, there is another less intense singlet at $\delta = 4.1$ ppm which can be attributed to the $-CH_2-$ group of the keto form; and moreover the ratio of the area of the phenyl signals to that of the olefinic proton is higher than 5.

The slow decrease of the signals at $\delta = 6.4$ and 4.1 ppm by addition of D_2O confirms their assignment to $-CH=$ and $-CH_2-$ groups and indicated that the keto-enolization rate is relatively slow.

In water the spectrum obtained by accumulation shows a predominance of the keto form (>95%); indeed near an intense multiplet between $\delta = 7.1$ ppm and $\delta = 7.4$ ppm, a region where both the signals deriving from all the protons of the phenyl ring of the keto form and those generated by the three protons of the phenyl ring of the enol form are found, only low signals due to the absorption of the olefinic proton ($\delta = 6.4$ ppm) and of the two other phenyl protons of the enol forms appear.

In Fig. 1 a the complete ^{13}C NMR spectrum of PPA in DMSO-d_6 is presented. The chemical shifts of the various carbon atoms were easily assigned on the ground of their analogy with the ^{13}C shifts of similar compounds [10]. The signals at 141.7 and

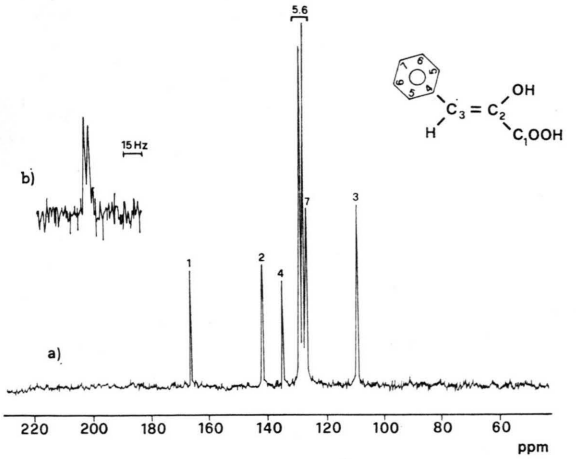


Fig. 1. a. ^{13}C NMR, ^1H noise decoupled, spectrum (25.2 MHz) of 1 M PPA in DMSO-d_6. Number of transients: 512, 1.25 Hz per data point. b. Expanded ^{13}C NMR spectrum of carboxyl carbon obtained from 0.5 M PPA in DMSO-d_6 without ^1H decoupling. Number of transients: 14.000, 0,68 Hz per data point.

109.9 ppm arising from olefinic carbons confirm unequivocally the predominance of the enol form in a DMSO solution of PPA.

Sodium phenylpyruvate spectra

The ^1H NMR spectrum of sodium phenylpyruvate in H_2O (Fig. 2) shows a strong resonance at $\delta =$ 4.07, near the solvent signal, derived from the $-CH_2-CO-$ protons and two small signals at $\delta =$ 6.3 and 3.05 ppm. This indicates that also the phenylpyruvate anion is present almost wholly in the keto

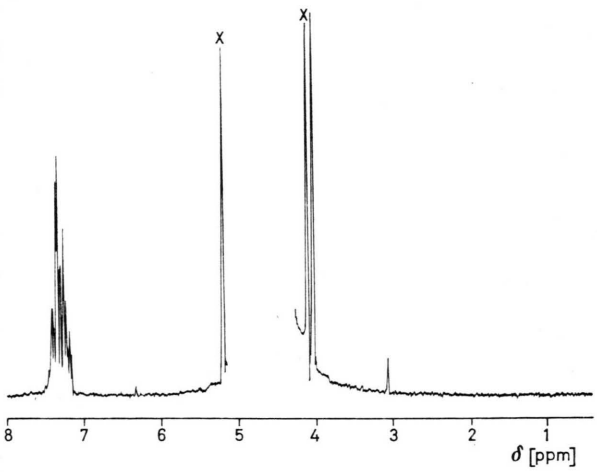


Fig. 2. Single scanned ^1H NMR spectrum (100 MHz) of a saturated solution (0.45 M) of sodium phenylpyruvate in H_2O. The X marks indicate spinning-sidebands.

form in this solvent. The resonance at $\delta = 6.3$ ppm is clearly due to the enolate anion, whereas the peak at $\delta = 3.05$ ppm may be due to traces of the hydrated form:

$$C_6H_5-CH_2-CO-COO^- + H_2O \rightleftarrows C_6H_5-CH_2-C(OH)_2-COO^-$$

on the basis of these considerations:

1. The ^1H NMR spectrum of sodium phenylpyruvate in D_2O shows a proportional lowering with time of the peak at $\delta = 4.07$ and 3.05 ppm, which is due to deuteration, resulting from the dynamics of the keto-enol equilibrium.
2. The relative chemical shift between these peaks (1 ppm) is similar to that observed for CH_3-CO- and $CH_3-C(OH)_2-$ groups in pyruvic acid [11].

It is impossible from the spectra of NaPP in H_2O to know the amounts of the forms present because the signal generated by the solvent is too near to that of $-CH_2-CO$ and prevents integration.

By measuring the integrated intensities of the aforesaid signals for freshly prepared solutions of NaPP in D_2O, the following poulation fractions were obtained:

$$X_{enol} = 2 \div 3\%$$
$$X_{diol} = 7 \div 8\%$$
$$X_{keto} = 89 \div 91\%.$$

The small amount of hydrated anion is due to the weak tendency of the carbonyl group to hydration since the electron withdrawing action of the carboxyl group is damped by deprotonation.

Same percentage were also obtained from a freshly prepared solution of PPA in phosphate buffer at pD = 6.

Tautomeric equilibrium of PPA

^1H NMR analysis appears valuable to prove that keto-enol tautomerism exists in solution for PPA.

The relative amounts of the enol and keto forms of PPA in a few organic solvents were straightforwardly determined by integrating the signals originating from the olefinic proton and the methylene protons (see Table II).

The small amount of enol form found in aqueous solution (0.005 M) cannot be estimated with sufficient precision by NMR techniques as saturation effects cannot be ruled out at such a low concentra-

tion. Therefore the values of the tautomeric equilibrium population in water were derived from optical data. The absorbance of the solution was measured at $\lambda = 295$ nm as at these wave-lengths the molar extinction coefficient of the enol form is several times higher than that of the keto form [12, 13].

It is assumed that the extinction coefficient of the enol PPA in water is of the same order as that in dioxane, methanol and 1 N HCl (see Table I). The choice of the two organic solvents was based on the ^{1}H NMR spectra results which show that the keto isomer is not present in these solvents.

Table I. Molar extinction coefficients of enol PPA at 25 °C.

Solvent	λ_{max}[nm]	ε
Dioxane	287	16,166
Methanol	292	23,567
1 N HCl	289	25,000

In Table II the values of the equilibrium constants $K_T = P_K/P_E$ in a few solvents at 25 °C determined by ^{1}H NMR and UV spectroscopy are reported. P_K and P_E represent the fractions of the keto and enol species, respectively.

Table II. Tautomeric equilibrium constants, K_T, of phenylpyruvic acid at 25 °C.

Solvent	K_T
Dioxane	~0
Methanol	~0
Acetone	$3.6_2 \pm 1.0_9 \times 10^{-2}$
DMSO	$7.5_3 \pm 1.1_6 \times 10^{-2}$
Water	19.00
1 N HCl	9.00[6]

Tautomeric equilibrium depends on the solvent properties and the low content of the enol form in aqueous solution agrees with the data found for other α keto acids, such as *p*-hydroxy-phenylpyruvic acid [12] and other pyruvic acid derivatives [11, 14].

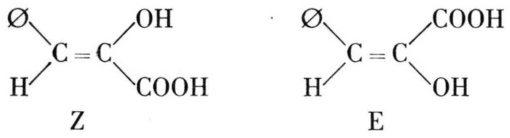
It is of interest that in protophylic solvents and in methanol the ratio of the tautomer amounts is inverted and PPA is more stable in its enol form.

Therefore in the limits of sensitivity of NMR spectroscopy, only this form is found in dioxane and methanol.

This different behaviour might be thus motivated:

1. the conjugation between the phenyl ring and the olefinic group makes the enol form more stable;
2. the less polar tautomer is favoured in the media of lower dielectric constant;
3. stabilization occurs by preferential solvation or hydrogen bonding between protophylic or amphiprotic solvents and the hydroxyl group of the enol form.

In theory, tautomerism should produce two enol isomers:



The presence of a single peak due to the olefinic proton, even in expanded spectra recorded for solutions in different solvents (DMSO-d_6, Acetone-d_6, CD_3OD and dioxane), and the fact that in ^{13}C NMR spectra (Fig. 1 a) (solvent: DMSO) two distinct signals for the carbon atom of the carboxyl group are not observed according to geometric isomerism point out the formation of only one enol form.

Evidence concerning the configuration of the enol tautomer present can be obtained from the value of the vicinal $^1H-C=C-{}^{13}COOH$ coupling constant. The dependence of the vicinal $^1H-C=C-{}^{13}C$ coupling constant on stereochemistry and on the nature of substituents has been investigated. It has been found that in substituted alkenes $^3J^{trans}_{^1H,^{13}C}$ is always larger than $^3J^{cis}_{^1H,^{13}C}$ [15, 16].

In the, ^{1}H coupled, ^{13}C NMR spectrum (Fig. 1 b) of PPA in DMSO, the resonance of the carboxyl carbon nucleous appears as a sharp doublet which reveals a coupling $^3J_{^1H,^{13}COOH} = 3.7$ Hz.

This value is in excellent agreement with those observed for the cis coupling constants in compounds with similar sums of substituent electronegativities [16]. Therefore, the Z configuration is attributed to the enol form of PPA.

Mass spectra of PPA

The existence of PPA almost completely in the enol form in methanol and dioxane and in the keto form in water encouraged the examination of residues obtained from these solutions by evaporation, under reduced pressure, of the solvents at room temperature.

The mass spectra (Fig. 3) of the residues coincide. The fragmentation pattern, accounting for the formation of all the main fragment ions, is reported,

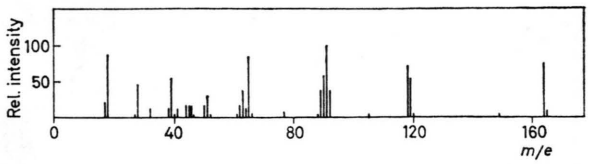
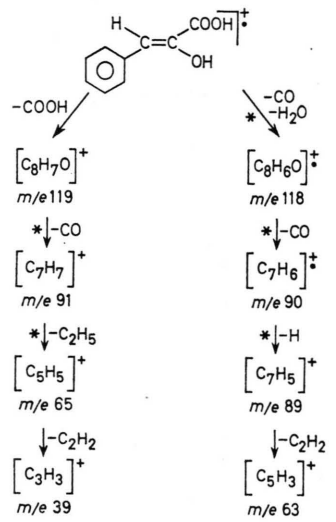


Fig. 3. Mass spectrum of the residue derived from a solution (0.05 M) of PPA in free peroxide dioxane. Electron beam energy: 70 eV; ion-accelerating voltage: 2 kV; probe temperature: 60 °C; electron multiplier sensitivity: 3×10; source pressure: 1×10^{-7} mm Hg.



Scheme 1. Fragmentation pattern. An asterisk indicates that a metastable peak was observed for that fragmentation.

in Scheme 1. It suggests that PPA dissociates largely by two competing paths.

Path I – The α-cleavage of the molecular ion ($m/e = 164$) gives rise to the ion m/e 119 as expected for carboxylic acids [17]. A metastable peak was not observed; however the formation of this ion seems to be so straightforward that its identity was not questioned. The loss of a CO molecule from the m/e 119 ion producing the base peak ion at m/e 91 and the subsequent fragmentation caracteristic of the tropilium [17, 18] ion are supported by the presence of two metastable peaks at m/e 69.5 ($119 \rightarrow 91$) and 46.4 ($91 \rightarrow 65$). A double charged ion at m/e 45.5 also appears.

Path II – The alternative mode of decomposition of the molecular ion starts with the loss of two neutral molecules CO and H_2O to give $[C_8H_6O]^+$ (m/e 118). A metastable ion at m/e 84.9 was observed. The $[C_8H_6O]^+$ ion, by eliminating a CO molecule, produces $[C_7H_6]^+$ (m/e 90) as proved by a metastable ion at m/e 69.5. The presence of another metastable peak at m/e 88.0 shows that $[C_7H_6]^+$ is the precursor of the ion with m/e 89. The decomposition modes confirmed by the presence of metastable peaks are listed in Table III.

Table III. Observed metastable decompositions.

[m*]	
46.4	$91^+ \rightarrow 65^+ + 26$
68.6	$118^+ \rightarrow 90^+ + 28$
69.5	$119^+ \rightarrow 91^+ + 28$
84.9	$164^+ \rightarrow 118^+ + 46$
88.0	$90^+ \rightarrow 89^+ + 1$

The modes for which a metastable decomposition was not observed can be justified with reference to the literature [17, 19] excepted the formation of the ion at $m/e = 92$.

The origin of this ion is not yet clear. Two nominal processes can account for its formation:

$$[C_9H_8O_3]^+ \xrightarrow{C_2O_3} [C_7H_8]^+ \text{ [20]}$$

$$[C_8H_6O]^+ \xrightarrow{C_2H_4} [C_6H_4O]^+$$

but no metastable ion was noticed in the spectrum for deciding between either of them.

IR spectra analysis

The infrared spectra (Fig. 4) of residues derived from solutions of PPA in aprotic solvents and methanol by slow evaporation of the solvents at room temperature are identical. A sharp band at

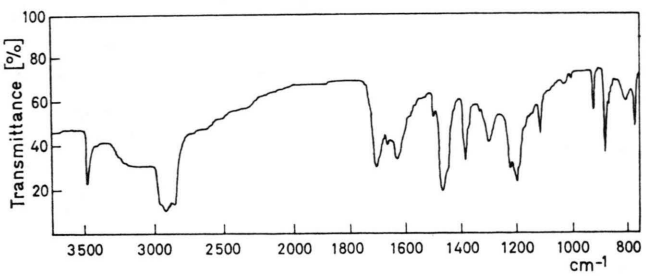


Fig. 4. Infrared spectrum in nujol mull of the residue derived from a solution (0.05 M) of PPA in free peroxide dioxane.

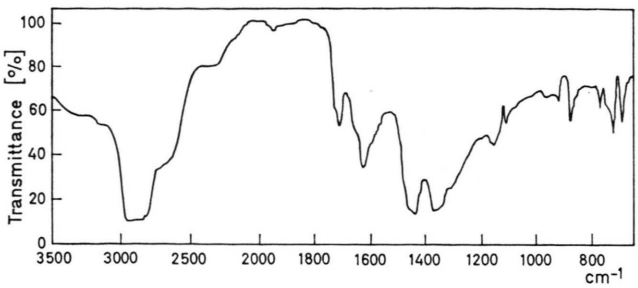


Fig. 5. Infrared spectrum in nujol mull of commercially available monohydrated sodium phenylpyruvate.

3470 cm^{-1} can be attributed to the stretching vibration of the free enol hydroxyl group [21]. A broad absorption band at 3000 cm^{-1} characteristic for the stretching vibration of the carboxyl group in hydrogen bonded dimers or carboxylic acids is also observed. A strong band at 1700 cm^{-1} can be assigned to the stretching vibration of the carboxyl C = O group in dimeric α, β unsatured acids [22, 23]. In fact, the keto form of PPA, which might be regarded as an aliphatic α keto acid, should have carboxyl C = O band shifted to higher frequencies because of the $-I$ effects of the adjacent carbonyl group. Indeed alkyl derivatives of pyruvic acid absorb in the region from 1725 to 1730 cm^{-1} [22]. A band at 1625 cm^{-1} is in the region of the C = C stretching vibration frequencies of a number of α, β unsatured acids [23] and is very near to that of cinnamic acid situated at 1620 cm^{-1}.

These data suggest that PPA in its crystalline form and the residues received after evaporation from organic solvents are in the enol form. The same IR spectrum was obtained from the residue of an aqueous solution. In this case slow evaporation of water causes continuous precipitation of the less soluble tautomer *i. e.* the enol form and hence conversion of the keto form to the other isomer.

IR spectrum of the sodium salt of PPA

The spectrum of sodium phenylpyruvate shows a medium intense band at 1710 cm^{-1} typical for keto carbonyl groups and a band at 1625 cm^{-1} deriving from the asymmetric stretching of the carboxylate group. Therefore it can be argued that the salt in the solid state is in the keto form (Fig. 5).

Analytical test with $FeCl_3$

The addition of ferric chloride traces to a PPA solution in DMSO yields an istantaneous development of a green colour. The ^1H and ^{13}C NMR spectra show that in such solutions only the enol form is present whereas both the keto form and eventual products of oxidation are absent. Thus it may be argued that the enol form of PPA does form a complex with ferric cation. The gradual appearance of the colour observed by many authors in water [4, 24] is probably due to the rate of enolization of the keto form. In DMSO the complex is stable in time on the contrary to what happens in water so that the use of the former solvent for PPA determination may be considered. These results suggest some changes in the colorimetric determination of PPA according to Berry and Woolf such as the use of peroxide free ether for its extraction and the use of DMSO as solvent to dissolve the dry acid.

The formula of the complex (2 : 1) was determined in DMSO by UV spectrophotometry (λ = 640 nm) according to the well-known Job's method [25].

[1] H. Bickel, F. P. Hudson, and L. J. Woolf, Phenylketonuria, G. Thieme Verlag, Stuttgart 1971.

[2] G. Weber, P. L. Glazer, and R. A. Ross, Adv. Enzyme Regulation **8**, 13 [1970]; J. A. Bowden and C. L. Mc Arthur III, Nature **235**, 230 [1972].

[3] E. Erlenmeyer jun., Ber. dtsch. Chem. Ges. **33**, 3001 [1900]; A. Following and K. Closs, Hoppe-Seyler's Z. Physiol. Chem. **254**, 115 [1938]; O. Meulemans, Clin. Chim. Acta **5**, 48 [1960].

[4] J. P. Berry and L. I. Woolf, Nature **169**, 202 [1952].

[5] M. L. Josien, M. Joussot-Dubien, and J. Vizet, Bull. Soc. Chim. France **24**, 1148 [1957].

[6] P. O. Larsen and E. Wieczorkowska, Acta Chem. Scand. **B 28**, 92 [1974].

[7] W. E. Knox and B. M. Pitt, J. Biol. Chem. **225**, 675 [1957].

[8] H. Strehlow, Z. Elektrochem. Ber. Bunsenges. physik. Chem. **66**, 392 [1962].

[9] K. Taniguchi and M. D. Armstrong, J. Biol. Chem. **238**, 4091 [1963].

[10] G. C. Levy and G. L. Nelson, Carbon-13 Nuclear Magnetic Resonance for Organic Chemists, Wiley-Interscience 1972.

[11] M. Becker, Z. Elektrochem. Ber. Bunsenges. physik. Chem. **68**, 669 [1964].

[12] T. Bücher and E. Kirberger, Biochim. Biophys. Acta **8**, 401 [1952].

[13] S. S. Tate, A. K. Grzybowski, and S. P. Datta, J. Chem. Soc. **1964**, 1372.

[14] A. Schellenberger and G. Hübner, Chem. Ber. **98**, 1938 [1965].

[15] K. M. Crecely, R. W. Crecely, and J. H. Goldstein, J. Mol. Spectrosc. **37**, 252 [1971]; J. L. Marshall and R. Seiwell, J. Magn. Resonance **15**, 150 [1974].

[16] J. A. Stubbe and G. L. Kenyon, Biochem. **10**, 2669 [1971]; U. Vögeli and W. von Philipsborn, Org. Magn. Resonance, in press.

[17] F. W. McLafferty and R. S. Gohlke, Anal. Chem. **31**, 2076 [1959]; S. Meyerson, J. D. McCollum, and P. N. Rylander, J. Amer. Chem. Soc. **83**, 1401 [1961]; A. G. Harrison, M. T. Thomas, and T. W. Still, J. Org. Mass. Spectrom. **3**, 899 [1970]; E. M. Emery, Anal. Chem. **32**, 1495 [1960].

[18] K. R. Jennings and J. H. Futrell, J. Chem. Phys. **44**, 4315 [1966]; S. Meyerson and P. N. Rylander, J. Chem. Phys. **27**, 901 [1957]; P. N. Rylander, S. Meyerson, and H. M. Grubb, J. Amer. Chem. Soc. **79**, 842 [1957].

[19] J. L. Franklin and S. R. Carroll, J. Amer. Chem. Soc. **91**, 5940 [1969].

[20] J. H. Beynon, G. R. Lester, and A. E. Williams, J. Phys. Chem. **63**, 1861 [1959].

[21] G. Nazario and K. Schwarz, Archiv. Biochem. Biophys. **123**, 457 [1968].

[22] M. Avram and Gh. Mateescu, Infrared Spectroscopy, Wiley-Interscience 1972.

[23] M. St. C. Flett, J. Chem. Soc. **1951**, 562.

[24] T. P. The, P. Fleury, and C. L. J. Vink, Clin. Chim. Acta **2**, 424 [1957]; M. Polonovski, P. Desgrez, and F. Delbarre, Bull. Soc. Chim. Biol. **29**, 1049 [1974]; G. A. Jervis, Proc. Soc. Exp. Biol. Med. **81**, 715 [1952].

[25] P. Job, Ann. Chim. (Paris) **9**, 113 [1926].