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The Ionization of Citric Acid Studied by the Nuclear Magnetic Resonance Technique[1]

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The chemical shift of the methylene hydrogens in citric acid was measured as a function of the pH and the concentration of the solution. From these measurements it was possible to evaluate the chemical shifts associated with each ionization step in citric acid. Measurements were also made of methylene resonance line positions for ionized and non-ionized methyl esters of citric acid. Analysis of the results indicates that the first and second ionizations of citric acid take place predominantly at the terminal carboxyl groups.

Introduction

Citric acid has three ionizable carboxyl groups and hence three dissociation constants, here denoted as pK_{A_1}, pK_{A_2} and pK_{A_3}. The monoionized citrate ion may exist in two forms, which are in equilibrium: in one form, the hydrogen of the carboxyl group α to the methylene group is ionized; whereas, in the second form, the hydrogen is ionized from the carboxyl group bonded to the tertiary carbon atom. In a similar way, two forms of the diionized citrate ion may exist. The purpose of this investigation was to evaluate the relative concentrations of the different species in solution using the n.m.r. technique.
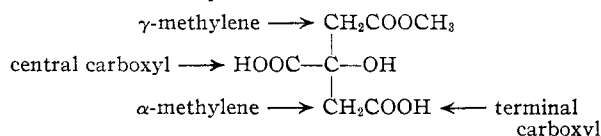
The n.m.r. spectrum (Fig. 1) of an aqueous solution of citric acid shows a strong doublet and two weak lines, each one symmetrically spaced on both sides of the doublet. These lines are due to the two methylene groups of citric acid and can be interpreted as being due to the non-equivalence of the two hydrogens in each methylene group.[3] The carboxylic, hydroxylic and water hydrogens exchange rapidly with one another and therefore appear as a single line.[4] As most of the experiments described in this paper involved measurements of chemical shifts, it was found convenient to measure all shifts relative to the resonance of a reference material added to the solution. The material chosen for this purpose was tetramethylammonium bromide (TMA),[5] which was added to all solutions in a concentration of about 0.2 M. A typical spectrum of an aqueous solution of citric acid, containing TMA but not showing the exchangeable hydrogens, can be seen in Fig. 1.

It was found that the chemical shift of the center of the strong methylene doublet (herein designated as δ) changes with the pH of the solution. This phenomenon is analogous to similar results obtained with other acids and bases.[5] From the variation of δ with the pH we have estimated the chemical shifts associated with each ionization step in citric acid. These measurements alone do not, however, provide sufficient information to evaluate the relative concentrations of the different ionic forms in the solution. To achieve this purpose, one or more of the exchangeable positions in citric acid has to be blocked with the minimum change in the rest of the molecule. The materials which appear to best serve this purpose are the methyl esters of citric acid. A series of measurements was therefore taken on the citric acid methyl esters. Analysis of the results obtained from citric acid and its methyl esters has enabled us to estimate the relative concentrations of the different ionized forms in the solution.

Notation

The following notations will be used to designate different methylene and carboxyl groups in citric acid and its methyl esters

$$\gamma\text{-methylene} \longrightarrow CH_2COOCH_3$$
$$\text{central carboxyl} \longrightarrow HOOC\text{—}\overset{|}{C}\text{—}OH$$
$$\alpha\text{-methylene} \longrightarrow \underset{|}{CH_2COOH} \longleftarrow \text{terminal carboxyl}$$

Using this notation, both methylene groups in citric acid are α-methylenes, whereas both methylene groups in the symmetrical dimethyl ester are γ-methylenes. The designations α and γ will be used irrespective of whether the carboxyl groups are ionized or not.

Experimental

The n. m. r. spectrometer used was the Varian model 4300 B operated at a proton frequency of 60 Mc. The frequency calibration was accomplished by the side-band technique. The precision of the measurements is expressed by the standard deviation, $\delta = [\Sigma d_i^2/(n-1)]^{1/2}$, where d_i represents the residual from the average value and n is the number of measurements (usually about 15).

The citric acid (A.R. monohydrate) solutions used in the n.m.r. measurements were prepared by titrating solutions of citric acid in hydrochloric acid (0.1–0.01 N) or in water, with a solution of tripotassium citrate in sodium hydroxide (0.01–1.0 N). Both acidic and basic solutions were of the same molarity in citrate ions and contained a constant concentration of TMA (about 0.2 M). The pH of the mixtures was measured with a Beckman model G pH meter using glass and calomel electrodes.

The trimethyl ester of citric acid was prepared by the procedure given by Donaldson[6]; m.p. 73–73.5°.

Anal. Calcd.: C, 46.15; H, 5.98. Found: C, 45.83; H, 6.24.

The n.m.r. spectrum of an aqueous solution of trimethyl citrate is shown in Fig. 2. The resonance lines from left to right (with increasing field) are attributed to the following groups: (I) the central O–CH₃, (II) the terminal O–CH₃,

(1) Supported by the Office of Naval Research.

(2) On leave of absence from the Weizmann Institute of Science, Rehovot, Israel.

(3) *Cf.* P. M. Nair and J. D. Roberts, This Journal, **79**, 4565 (1957).

(4) E. L. Hahn and D. E. Maxwell, *Phys. Rev.*, **88**, 1070 (1952); H. S. Gutowsky, D. W. McCall and C. P. Slichter, *J. Chem. Phys.*, **21**, 279 (1953).

(5) E. Grunwald, A. Loewenstein and S. Meiboom, *ibid.*, **27**, 641 (1957).
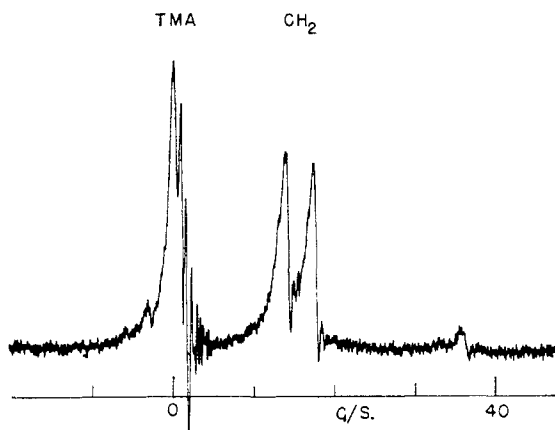
(6) W. E. Donaldson, R. F. McCleary and E. F. Degering This Journal, **56**, 459 (1934).

Fig. 1.—N.m.r. spectrum of aqueous solution of citric acid (~ 1 M) at pH ~ 2. Tetramethylammonium bromide (TMA) was added as a reference compound to all solutions. The quadruplet (a strong doublet and two weak satellites) due to the non-equivalent methylene hydrogens is clearly resolved. In this and subsequent figures the field intensity increases from left to right, and the resonance of the exchangeable (acid, hydroxyl and water) protons is not recorded.
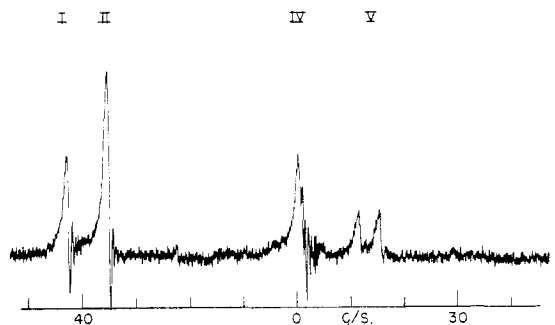

Fig. 2.—N.m.r. spectrum of an aqueous solution of trimethyl citrate. The resonance lines are attributed to the groups: (I) central-OCH₃, (II) terminal-OCH₃, (IV) TMA, (V) γ-CH₂.

(IV) the TMA and (V) the γ-methylene. The resonance line of the exchangeable hydrogens is not shown. It should be noted that the satellite lines expected on both sides of the doublet V are barely observable because of their low intensity. The same will apply to the resonance of all the methylene groups shown in succeeding figures.

Citric acid symmetrical dimethyl ester was prepared according to the procedure given by Schroeter[7]; m.p. 115–117°.

Anal. Calcd.: C, 40.30; H, 5.88. Found: C, 40.00; H, 5.75.

This material is barely soluble in water; therefore, its n.m.r. spectrum is recorded with difficulty. Nevertheless the weak spectrum obtained was in complete agreement with the expectations for the assigned structure. Citric acid symmetrical dimethyl ester dissolves easily in one mole of base to give the symmetrical dimethyl citrate. The spectrum of this solution shows one CH₂ doublet and one O–CH₃ line.

Citric acid unsymmetrical monomethyl ester was obtained in solution by basic hydrolysis of one mole of symmetrical dimethyl ester with two moles of aqueous sodium hydroxide. The n.m.r. spectrum of the solution, taken immediately after preparation, is presented in Fig. 3. The resonance of the exchangeable hydrogens is not shown.

The resonance lines shown in Fig. 3, from left to right (with increasing field), are attributed to the following groups: (I) a central OCH₃ group (due to a small amount of sym-

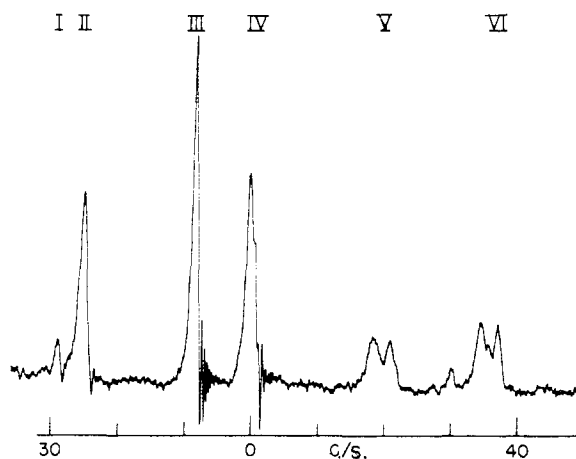(7) G. Schroeter, *Ber.*, **38**, 3190 (1905).

Fig. 3.—N.m.r. spectrum of a solution of 1 mole of symmetrical dimethyl citrate in two moles of base. The hydrolysis product is unsymmetrical monomethyl citrate. The resonance lines are attributed to these groups: (I) central-OCH₃, (II) terminal-OCH₃, (III) methanol, (IV) TMA, (V) γ-CH₂, (VI) α-CH₂.

metrical monomethyl ester), (II) the terminal OCH₃ group in the unsymmetrical monomethyl ester, (III) the methyl group in methanol which is a by-product of the hydrolysis (IV) TMA, (V) the γ-methylene of the unsymmetrical monomethyl ester and (VI) the α-methylene of the unsymmetrical monomethyl ester. The above notation of the lines will be used throughout for the undissociated esters and their corresponding anions.

The evidence presented supports the above interpretation of the spectrum: (1) lines corresponding to I and II are observed in ratio of 1:2 in the spectrum of trimethyl ester of citric acid at approximately the same positions relative to TMA (see Fig. 2); (2) when methanol is added to the solution line III grows in intensity, but on reduced pressure evaporation of the solution and re-solution in water, line III disappears; (3) line V appears at approximately the same position relative to TMA as the γ-methylene resonances in the di- or trimethyl esters; (4) when sodium citrate is added to the solution doublet VI increases in intensity, this being due to the addition of α-methylene groups of citrate ion; and (5) addition of more base to the solution results in decreasing the intensity of lines I, II and V, while lines VI and III grow in intensity. This can be explained as being due to further hydrolysis of the monomethyl ester to citric acid and methanol.

Citric acid symmetrical monomethyl ester was obtained in solution by the basic hydrolysis of the trimethyl ester. One mole of trimethyl ester was dissolved in two moles of aqueous sodium hydroxide. The n.m.r. spectrum of this solution is shown in Fig. 4. The resonance of the exchangeable hydrogens is not included in the figure, and the same notation for the lines is used as for Figs. 2 and 3. The fact that the rapid hydrolysis gives mainly the symmetrical monomethyl ester is proved by the appearance of only one doublet (VI), which means that both methylene groups are located symmetrically with respect to the remaining esterified carboxyl group.

It can be concluded from these experiments that the hydrolysis of the di- and trimethyl esters to the monoesters is fast and complete within the time taken between preparation of the solution and recording its n.m.r. spectrum (about 10 minutes). This agrees with previous observations.[8]

Results

As mentioned in the Introduction, the chemical shift (δ) between the strong doublet in citric acid and TMA changes with the pH of the solution. A plot of δ as function of the pH is shown in Fig. 5.

Figure 5 has the characteristics of an acid–base titration curve. Since, however, citric acid solu-

(8) A. Skrabal, *Z. Elektrochem.*, **33**, 332 (1927).

TABLE I

METHYLENE CHEMICAL SHIFTS MEASURED IN C.P.S. RELATIVE TO TMA

	Citric acid α-CH$_2$	Unsymmetrical monoester		Symmetrical monoester α-CH$_2$	Symmetrical diester γ-CH$_2$	Triester γ-CH$_2$
		α-CH$_2$	γ-CH$_2$			
Un-ionized	13.3 ± 0.2	13.3 ± 0.7	13.3 ± 0.7	13.3 ± 0.6	........[a]	13.3 ± 0.6
Fully ionized	35.9 ± 0.2	35.9 ± 0.4	20.7 ± 0.2	32.6 ± 0.6	$20.4 \pm 0.7^{[b]}$	....

[a] Not measured due to insolubility.　[b] No figure shown for spectrum.

tions are strongly buffered and the pK_A values do not differ much from each other, the inflection points in the titration curve of the tribasic acid are not clearly resolved. Hence, the chemical shifts associated with each ionization step cannot be directly read from Fig. 5, and a different procedure must be used. Another feature of Fig. 5 is the independence of δ of the concentration of the acid. The significance of this observation will be discussed later.
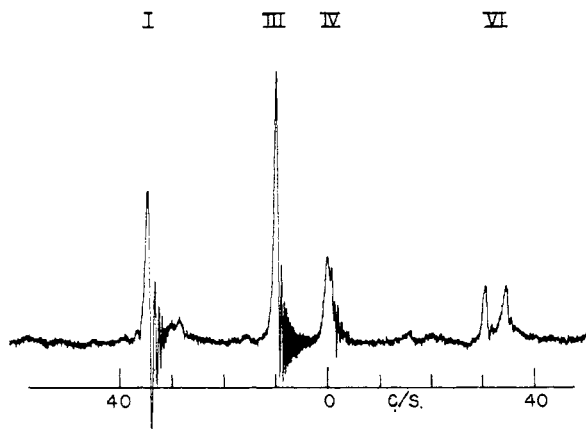
Fig. 4.—N.m.r. spectrum of a solution of 1 mole of trimethyl citrate in two moles of base. The hydrolysis product is symmetrical monomethyl citrate. The resonance lines are attributed to these groups: (I) central-OCH$_3$, (III) methanol, (IV) TMA, (VI) α-CH$_2$.

The spectrum of the diionized, unsymmetrical monomethyl ester of citric acid was shown in Fig. 3. When two equivalents of acid are added to the solution of the diionized, unsymmetrical monomethyl ester, remarkable changes are observed in the spectrum as is seen in Fig. 6. This spectrum corresponds to the formation of un-ionized monomethyl ester in which the α- and γ-methylene resonances coincide.

The spectrum of an acidified solution of the symmetrical monomethyl ester shows only a shift in the position of the doublet VI.

The chemical shifts as measured from Figs. 2–6 are given in Table I.

Interpretation of the Data

The results were interpreted in two steps: (1) evaluation of the chemical shifts associated with each ionization step in citric acid and (2) evaluation of the relative concentrations of the different forms of the mono- and diionized citrate ions.

(1) The observed chemical shifts, δ, in citric acid can be described by the equation

$$\delta = x_1\delta_1 + x_2\delta_2 + x_3\delta_3 + x_4\delta_4 \qquad (1)$$

where x_1, x_2, x_3 and x_4 represent the mole fractions of the non-, mono-, di- and triionized citrates, respectively, and δ_1, δ_2, δ_3 and δ_4 their chemical shifts

relative to TMA. The use of equation 1 is justified since the various citric acid species in the solution are rapidly equilibrated by the very fast exchange of the carboxyl hydrogens. Equivalent expressions have been used previously in n.m.r. studies of systems which consist of several species in equilibrium.[9]

Fig. 5.—The chemical shift δ between the center of the strong methylene doublet in citric acid and TMA resonance, as function of the pH.

Fig. 6.—N.m.r. spectrum of an acidified solution of unsymmetrical monomethyl citrate (alkaline spectrum shown in Fig. 3). Line notations are same as in Fig. 3. The α- and γ-methylenes resonances here coincide.

RELATIVE CHEMICAL SHIFTS:

Zero   A   B   C

Fig. 7.—Schematic representation of the different ionic forms of citric acid and the notations used in calculating their relative concentrations. The calculated chemical shifts of the mono-, di- and triionized citric acid, relative to the non-ionized acid, are indicated as A, B and C.

The values of δ_1 and δ_4 can be taken directly from Fig. 5 or Table I since they are values of δ at very low and very high pH, respectively. Thus δ_1 = 13.3 ± 0.2 c./s. and δ_4 = 35.9 ± 0.2 c./s. The values of δ_2 and δ_2 have bee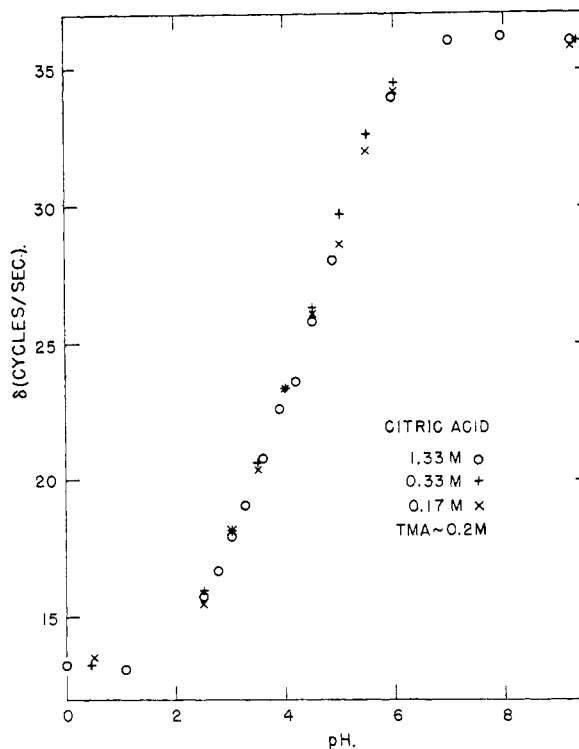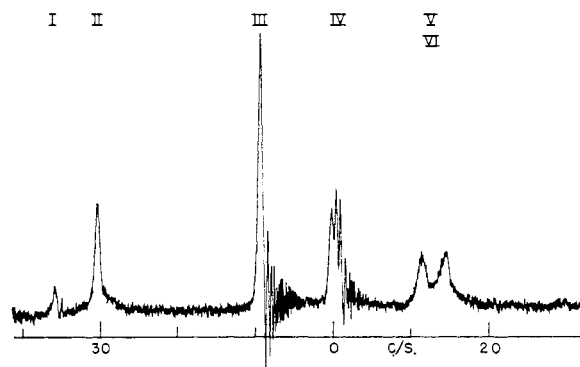n calculated so as to give the best fit with the titration curve presented in Fig. 5. For the computation of x_1, x_2, x_3 and x_4 for each pH, these pK_A values were used[10]: pK_{A_1} = 3.13, pK_{A_2} = 4.76, pK_{A_3} = 6.40.

The procedure used in the calculation of δ_2 and δ_3 from the data given in Fig. 5 is given: equation 1 was set up numerically for each point on the titration curve, using the experimental values of δ, δ_1 and δ_4 and the calculated values of the mole fractions (x_i). This gave one equation for each point on the titration curve that included δ_2 and δ_3 as unknowns. All possible pairs of such equations then were used to solve for δ_2 and δ_3. In practice, only the measurements in the pH range of 3–5.5 were chosen for the computation since the change of δ with the hydrogen ion concentration at a pH lower than 3 or higher than 5.5 was small and was measured with insufficient accuracy for this type of calculation. The average results thus obtained from the measurements taken with the 0.33 and 0.17 M solutions are: δ_2 = 22.5 ± 0.2 c./s.[11] and δ_3 = 33.5 ± 0.2 c./s. The procedure described above gives a minimum to $\Sigma(\delta_i - \Delta_i)$ calculated for *all* the points on the curve, where Δ_i are the calculated values of δ using the values of δ_2 and δ_3 given above.

Another procedure to calculate δ_2 and δ_3 was carried out: Different values of δ_2 and δ_3 were chosen by a "trial and error" procedure with the purpose of minimizing $\Sigma(\delta_i - \Delta_i)^2$. The best values for δ_2 and δ_3 thus obtained were δ_2 = 23.5 c./s. and δ_3 = 33.5 c./s. The result was identical for δ_3 and slightly

(9) See for example: M. Saunders and J. B. Hyne, *J. Chem. Phys.*, **29**, 1319 (1958).

(10) R. G. Bates and G. D. Pinching, THIS JOURNAL, **71**, 1274 (1949).

(11) The precision given here is not the standard deviation but the average deviation, $\Sigma|\delta_i - \Delta_i|/n$, where n is the number of points on the curve. It should be noted that the method of least squares can not be applied successfully to this problem because of the particular form of the function $\delta(x_i, \delta_i)$.

RELATIVE CHEMICAL SHIFTS:

Zero α-CH$_2$: D   Zero   F
        γ-CH$_2$: E

Fig. 8.—Schematic representation of monomethyl esters of citric acid and notations used in calculations. The measured chemical shifts, relative to non-ionized citric acid, are indicated as D, E and F.

different for δ_2 as compared to the previously described procedure. The value of δ_2 = 22.5 c./s., however, is preferred since by using it the calculated δ-values are more equally scattered (positive and negative deviations) along the experimental curve.

The chemical shifts associated with each ionization in citric acid therefore are

single ionization: $\delta_2 - \delta_1 = 22.5 - 13.3 = 9.2 \pm 0.3$ c./s.

double ionization: $\delta_3 - \delta_1 = 33.5 - 13.3 = 20.2 \pm 0.3$ c./s.

triple ionization: $\delta_4 - \delta_1 = 35.9 - 13.3 = 22.6 \pm 0.3$ c./s.

It should be noted that both the use of equation 1 and the pK_A values cited above are supported by the fact that the chemical shifts, within the experimental accuracy, are independent of the concentration. If more than four terms contribute to δ in equation 1 (*i.e.*, presence of associated species in the solution) or if the K_A values were not applicable at the concentrations measured, one would expect δ to be concentration dependent.

(2) Figures 7 and 8 illustrate the chemical shifts, species and notations involved in the calculation of the relative concentrations of the ionized forms.

The symbols x, y, u and v are the mole fractions of the ionized species of citric acid shown in Fig. 7, such that $x + y$ equals unity for the monoionized acid and $u + v$ equals unity for the diionized acid. In addition, we define the following shifts measured relative to the methylene line of the non-ionized acid taken as zero

a = chemical shift of a methylene group located between a non-ionized, terminal carboxyl group and an ionized, central carboxyl group

b = chemical shift of a methylene group located between an ionized, terminal carboxyl group and a non-ionized, central carboxyl group

c = chemical shift of a methylene group located between two (central and terminal) ionized carboxyl groups

The following assumptions were made and are included in Figs. 7 and 8: (a) The methylated carboxyl group behaves with regard to the chemical shift as if it were a non-ionized carboxyl group. This assumption is supported by the observation that in all the non-ionized methyl esters only one doublet is observed and its chemical shift is the same as for non-ionized citric acid.[12] (b) The

(12) This observation could also be explained by assuming fast exchange of the alkyl radical of the ester groups between possible positions; this, however, is not considered to be probable.

chemical shift of a methylene group is not affected much by the terminal carboxyl located at the other end of the molecule, whether ionized or not. The validity of this assumption has been verified by the measurements of a and c in different species.

We proceed now to evaluate a, b and c using the data given in Table I. From the data given for the diionized, unsymmetrical monomethyl ester, we obtained the values for a and c since

$$a = E = 20.7 - 13.3 = 7.4 \pm 0.7 \text{ c./s.}$$

and

$$c = D = 35.9 - 13.3 = 22.6 \pm 0.8 \text{ c./s.}$$

The value of c also can be obtained from the measured chemical shift in triionized citrate since

$$c = C = 35.9 - 13.3 = 22.6 \pm 0.3 \text{ c./s.}$$

which agrees with the value obtained above.

The value of a can also be obtained from the chemical shift of the γ-methylene group in the ionized, symmetrical dimethyl ester, which gives $a = 20.4 - 13.3 = 7.1 \pm 0.7$ c./s. The small difference between this value and the value obtained above from the unsymmetrical monoester ($a = 7.4$ c./s.) may reflect the shift due to ionization in the far-end carboxyl group (in the unsymmetrical monoester) or be due to experimental inaccuracy. An average value, $a = 7.25 \pm 0.5$ c./s., will be used in further computations.

The value of b was obtained from the shift of the methylene group in the fully ionized, symmetrical monoester since

$$b = F = 32.6 - 13.3 = 19.3 \pm 0.8 \text{ c./s.}$$

One might also obtain b from the methylene shift in the ionized, unsymmetrical dimethyl ester; this compound, however, was not prepared. Hydrolysis of the triester with one mole of base gave a complex mixture of several hydrolysis products, together with non-hydrolyzed material.

The values obtained for a, b and c show that the strongest effect on the chemical shift of the methylene group is caused by ionization in the terminal carboxyl group, whereas ionization in the central carboxyl group is less effective. This is to be expected since one more bond separates the methylene group from the central carboxyl as compared to the terminal carboxyl.

Two equations can now be set up which will describe the observed chemical shifts in the mono- and diionized citrate ions

$$ax + by/2 = A \tag{2}$$
$$bv + (a + c)u/2 = B \tag{3}$$

and by definition

$$x + y = 1 \tag{4-a}$$
$$u + v = 1 \tag{4-b}$$

Equations 2 and 3, like equation 1, are based on the chemical equilibrium existing between the ionic species as a result of fast exchange of the hydrogens.

Substituting the values of a, b and A in equation 2, we obtain $7.25x + 9.65y = 9.2$. With the aid of equation 4-a we can solve for x and y, the result being

$$x = 0.2 \pm 0.2 \text{ and } y = 0.8 \pm 0.2$$

This means that the monoionized citrate ion is about 80% in the unsymmetrical form.

From numerical substitution in equation 3 we obtain $19.3v + 14.9u = 20.2$. This result obviously is inconsistent with equation 4-b and the demand that v and u should be positive. However the magnitude of the inconsistency is reduced to 0.9 c.p.s. (and not far beyond our experimental error) if v is taken to be unity, which means that the diionized acid is solely in the symmetrical form.[13] It is very possible that besides experimental error, the observed inconsistency of the results arises from *slight* differences in the values of a and b between the doubly ionized acid and the methyl esters. Such differences will be critical since it will be remembered that a and b were assumed to have the same values in all comparable species. Differences in a and b might arise from molecular association and hydrogen bonding which should play a more important role in the highly ionized species than in the esters.

The method of treatment of the data for citric acid was checked successfully through an analogous set of measurements and computations using succinic acid and its monomethyl ester. The results are presented in Appendix A.

Several factors may be effective in determining the location of the non-ionized protons: (1) a statistical factor of two will favor the unsymmetrical forms of the mono- and diionized species over the symmetrical forms; (2) maximum separation of the charges will lower the electrostatic interaction energy (this is important for the diionized ion and will tend to localize the undissociated proton on the central carboxyl group); (3) the alcoholic hydroxyl and the two methylene groups attached to the tertiary carbon will be operating as electron-attracting groups (negative inductive effect) and, thereby, should promote ionization of the central relative to the terminal carboxyl groups; and (4) if the hydroxyl group is intramolecularly hydrogen bonded to the terminal carboxyl groups, then ionization of these groups will strengthen the hydrogen bond, thus lowering the total energy.

The experimental results indicate that the balance of the above (or other) effects is to favor both initial and secondary ionization at the terminal carboxyl groups. Which effects are the more important is not now known.

Appendix A
Succinic Acid and Monomethyl Succinate.

Measurements on succinic acid and its methyl ester were made to provide a check on the calculations and on some of the results obtained for citric acid.

From a titration curve similar to that shown in Fig. 5 for citric acid the chemical shifts associated with the first and second ionizations in succinic acid were obtained. The procedure used to analyze the curve was the same as for citric acid. Equation

(13) Calculation of u and v gives: $v = 1.2 \pm 0.2$, and $u = -0.2 \pm 0.2$.

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