Acylation of Ascorbic Acid in Water

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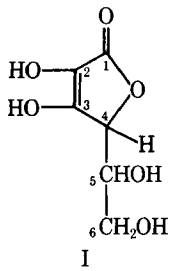
Abstract □ The acylation of ascorbic acid by acetic anhydride was studied in water at 25°. The results indicate that the initial products of reactions between these two compounds were acetic acid and 3-*O*-acetylascorbic acid. The latter product then underwent two parallel reactions: hydrolysis to ascorbic and acetic acids and an intramolecular O → O acyl migration to yield 2-*O*-acetylascorbic acid. The former reaction was predominant at pH values below 4, whereas the latter reaction predominated at pH values between 4 and 7. These results are used as a basis for questioning the structural assignments previously made to some ascorbic acid esters.

Keyphrases □ Ascorbic acid—acylation by acetic anhydride in water, kinetics and products *via* hydrolysis and rearrangement □ Acylation of ascorbic acid in water using acetic anhydride—kinetics and products *via* hydrolysis and rearrangement

A kinetic and product analysis is described for reactions that occur in aqueous solutions initially containing ascorbic acid (I) and acetic anhydride. The purposes of this study were to determine which hydroxy groups of I were acetylated and in what proportion and then to investigate the stability of the formed esters with respect to intramolecular O → O acyl transfers and hydrolysis. Intramolecular O → O acyl transfers were previously reported to occur for ascorbic acid esters (1), and they are well-known reactions of glycerol esters (2) and hydroxyacids (3, 4). The results of these studies will prove useful in the characterization of the structures of mono-, di-, and triacyl derivatives of ascorbic acid existing in water and in understanding the reactivity of these derivatives with respect to hydrolysis and rearrangement.

Considerable interest exists in the synthesis and properties of acyl derivatives of I. The major reasons for this interest are: *(a)* the resistance of ascorbic acid to autoxidation can be increased by esterifying one or both of the hydroxy groups in the enediol system [—(HO)C=C(OH)—] (1), and *(b)* esters can be conferred with almost any desired amount of lipophilic character by appropriate selection of acyl moieties.

Two advantages that esters of ascorbic acid of I might possess are that lipophilic derivatives can be included in nonaqueous preparations [*e.g.*, the use of 6-*O*-palmitoylascorbic acid (6) or 6-*O*-stearylascorbic acid (7) as antioxidants in nonaqueous formulations] and that they may be absorbed percutaneously more efficiently (8)[1]. Administration of ascorbic acid through the skin has been suggested (9) as a means of facilitating wound healing. However, it is believed (10) that both the antioxidant properties and antiscorbutic activity of I depend on the presence within the molecule of the enediol system. Consequently, it is important to know whether a particular ester contains an intact enediol system or whether the intact enediol system will be regenerated in the biological environment.



I

EXPERIMENTAL

Equipment—Spectrophotometric[2] and spectropolarimetric[3] studies were performed using instruments with thermostated cell compartments. All thermostated baths were regulated within ±0.1°.

Reagents—All reagents used were of analytical grade, unless otherwise stated. Acetic anhydride was distilled over magnesium turnings, and the fraction boiling within 137.8–138.2° was collected. Ascorbic acid was recrystallized from its concentrated solution in hot methanol by adding sufficient petroleum ether to make the solution turbid and keeping the resulting mixture overnight at room temperature.

The water used was redistilled from acid permanganate using an all-glass still. 5,6-Isopropolidine-2,3-di-*O*-acetylascorbic acid was synthesized following the Vestling and Rebstock (11) procedure. Unless otherwise mentioned, all experiments were carried out in aqueous solutions.

Measurement of Acetylation—Three milliliters of a solution of I (0.05 *M*) was combined with 2.0 ml of water and titrated with a standard solution of sodium hydroxide to the phenolphthalein end-point. The volume of the required titrant, *a* ml, was noted. Three milliliters of a solution of I (0.05 *M*) was reacted with 0.3 ml of 0.6 *M* solution of acetic anhydride (in dioxane) in a closed vial for 1 hr at 25°. The reaction mixture was then diluted with 2.0 ml of water and titrated to the same end-point. The volume of the titrant, *b* ml, was likewise noted. An equivalent amount of acetic anhydride was titrated after hydrolysis in 5.0 ml of water to the same end-point. The volume of the consumed titrant, *c* ml, was also recorded.

The ratio of ester to acetic anhydride and the percent acetylation were calculated from *a*, *b*, and *c* according to:

$$\frac{\text{ester}}{\text{acetic anhydride}} = \frac{(a + c) - b}{c} \times 2 \qquad \text{(Eq. 1)}$$

$$\%\ \text{acetylation} = \frac{(a + c) - b}{c} \times 100 \qquad \text{(Eq. 2)}$$

Determination of k_1^{obs}—The second-order rate constant (k_1^{obs}) for the initial attack of anhydride on ascorbic acid (Scheme I) was determined by following the loss of UV absorption at 275 nm. The theory is described under *Results and Discussion*. Acetate buffers (2×10^{-1} *M*) containing 1×10^{-4} *M* edetate (to minimize any ca-

[1] This reference describes an absorption study done with ascorbic acid 3-phosphate.

[2] Using Cary 15 and Cary 16 spectrophotometers, Varian Instruments, Inc.

[3] Using a Cary 60 spectropolarimeter, Varian Instruments, Inc.

talysis of the oxidation of ascorbic acid by trace elements) were used in these runs.

The solutions were adjusted to an ionic strength of 1.0 M with potassium chloride, and temperature was maintained at 25.0 ± 0.1°. The amount of ascorbic acid used was such as to give an initial absorption of about 0.9 unit (5×10^{-5} M at pH > 4.5, $1–2 \times 10^{-4}$ M at pH < 4.5). The amount of acetic anhydride added (as a solution in dioxane) was sufficient to produce a 100–400-fold excess of acetic anhydride concentration over that of ascorbic acid.

The loss of absorbance at 275 nm was recorded for about 1 min. About 20 sec (accurately measured) elapsed between mixing the reagents and taking the first reading in the spectrophotometer. The smoothness of the absorbance curve after about 15 sec indicated that the 20 sec allowed for the first point was sufficient for an accurate measurement of absorbance.

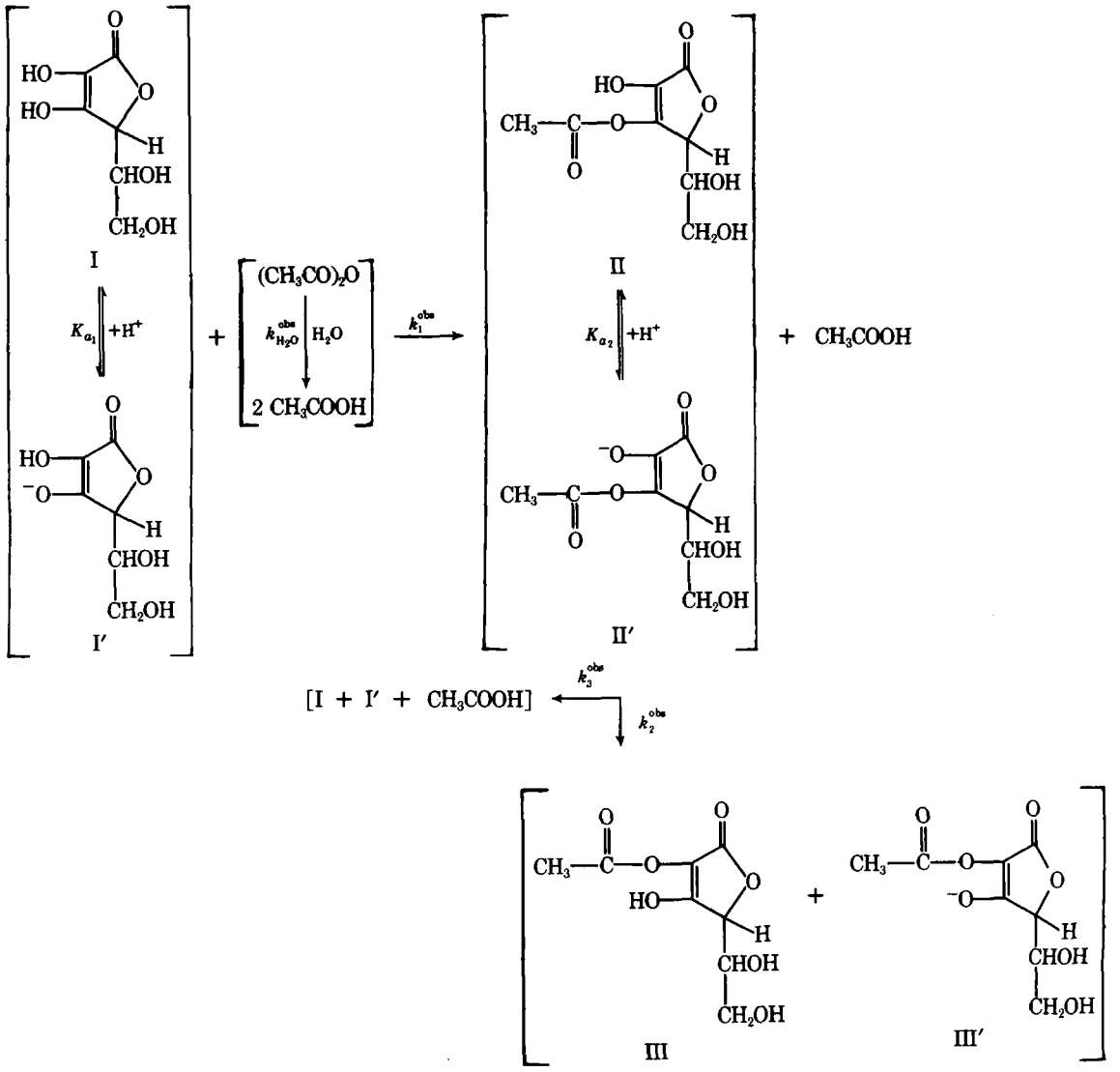
Determination of k_2^{obs}—The first-order rate constants for the loss of 3-*O*-acetylascorbic acid were determined by following the increase of UV absorption at the λ_{max} of 2-*O*-acetylascorbic acid. Citrate buffers (0.01 M) were used to minimize any catalysis of oxidation of ascorbic acid by trace elements (citrate complexes with the metal ions) and also to utilize the broad range of the buffering potential of the citric acid system. The solutions were adjusted to an ionic strength of 0.1 M with potassium chloride, and temperature was maintained at 25.0 ± 0.1°.

Concentrated solutions of 3-*O*-acetylascorbic acid formed *in situ* (see *Results and Discussion*) were diluted in the specified buffers (final concentration of ascorbic acid and its derivatives about 5×10^{-5} M–1×10^{-4} M), and the increase in UV absorbance was recorded until a plateau value was reached. Similarly, hydrolysis rate constants of 5,6-isopropolidine-2,3-di-*O*-acetylascorbic acid were determined by diluting a concentrated methanolic solution in the specified buffers and following the increase in UV absorbance at the λ_{max} of the 3-*O*-acetyl species.

RESULTS AND DISCUSSION

Several pieces of evidence suggest that the major series of reactions that occur when acetic anhydride (initial concentration $1–5 \times 10^{-2}$ M) is added to aqueous solutions of I (initial concentration $1–50 \times 10^{-2}$ M) at 25° are those shown in Scheme I. A qualitative picture of the events following the addition of acetic anhydride to solutions of I comes from the changes of optical rotation (at 350 nm) of the reaction solution as a function of time (Fig. 1). At pH values between 2 and 7, the changes in optical rotation were characterized by an initial rapid decrease followed by a slower increase. At pH values above 4.0, the final optical rotation was greater than the initial value. At lower pH values, the initial and final optical rotation values were similar.

It is believed that the initial decrease in optical rotation is caused by the conversion of I to 3-*O*-acetylascorbic acid (II) and



Scheme I

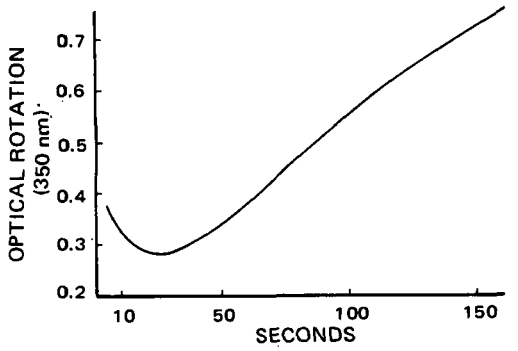


Figure 1—*Change in optical rotation at 350 nm as a function of time for the reactions of acetic anhydride (initial concentration 0.02 M) in a solution of ascorbic acid (initial concentration 0.25 M) (0.2 M acetate buffer, pH 5.25, I = 0.5 M, T = 27°).*

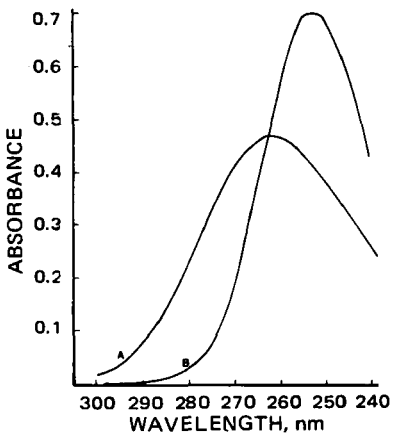


Figure 2—*UV spectra of: A, 5 × 10⁻⁵ M solution of ascorbic acid; and B, reaction mixture containing 5 × 10⁻⁵ M ascorbic acid and 1 × 10⁻² M acetic anhydride after 40 min. The solvent for both solutions was a 1 × 10⁻¹ M acetate buffer at pH 4.65.*

that the subsequent increase in optical rotation results from both the rearrangement of II to 2-*O*-acetylascorbic acid (III) *via* an O → O acyl migration and hydrolysis of II to I and acetic acid. These conclusions concerning the reaction mechanism are in part based on the assumption that the rate-determining step in the acylation reaction in water involves a reaction between an anionic form of I and acetic anhydride. It was previously found (4) that neutral molecules of alcohols, phenols, and hydroxycarboxylic acids are not significantly acylated by acetic anhydride in water whereas their anions, at least in the case of hydroxycarboxylic acids, are rapidly acetylated.

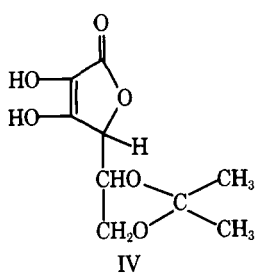
Hence, because the 3-hydroxy group is the most acidic in I (12), it is expected that it would be the first to be acetylated between pH 2 and 7. The large change in optical rotation that accompanies the initial reaction is consistent with the proposed reaction sequence because the 3-hydroxy group is adjacent to a rigid asymmetric carbon atom (C-4) and its derivatization would be expected to result in a relatively large change in optical activity. Additional evidence for this first reaction was obtained from a kinetic study and will be discussed later.

If III were simply hydrolyzed in a subsequent reaction to yield I and acetic acid, the initial and final optical activities of the reaction solutions would be the same. However, apart from the fact that the initial and final optical activities did differ by a small but significant amount, several other pieces of evidence indicate that the final reaction mixture contained at least one compound in addition to I (and I') and acetic acid. Evidence for this conclusion is:

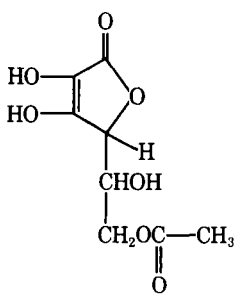
1. The UV spectrum of the initial and final reaction solutions differed significantly (Fig. 2).

2. Titration of the final reaction mixture with sodium hydroxide to the phenolphthalein end-point yielded a smaller titer than that obtained for a mixture containing ascorbic acid and fully hydrolyzed acetic anhydride. For example, 4.8 ml of 0.1 *N* NaOH was required to titrate 3.3 ml of a reaction mixture that originally contained 0.15 m*M* I and 0.18 m*M* acetic anhydride and had stood at 25° for 1 hr. If each mole of acetic anhydride had reacted to yield 2 moles of acetic acid, the titer would have been 5.1 ml.

The major product following these reactions is believed to be III rather than the 5-*O*-acetyl or 6-*O*-acetyl derivatives of ascorbic acid for several reasons. First, 5,6-isopropolidineascorbic acid (IV) and 6-*O*-acetylascorbic acid (V) reacted in a similar manner (both qualitatively and quantitatively) to I in aqueous solutions of acetic



IV



V

anhydride (the 6-hydroxy group cannot be acetylated in either compound and both the 5-hydroxy and 6-hydroxy groups are masked in IV). Second, potentiometric titration of the final reaction mixture of I and acetic anhydride with hydrochloric acid revealed that it contained a compound with a pKa of approximately 2 [it seems unlikely that 5-*O*-acetyl- or 6-*O*-acetylascorbic acid would have significantly different pKa values from that of I (pKa = 3.90 at I = 0.10 *M*)]. And third, titration of a 1-hr-old reaction mixture, made by adding 0.1 ml of a 0.6 *M* solution of acetic anhydride in dioxane to 3.0 ml of a 0.1 *M* solution of sodium ascorbate (at pH 6.0) with 2,6-dichloroindophenol or iodine, gave titers accounting for only between 82 and 84% of the original amount of ascorbic acid. Since both of these reactants detect molecules that contain an intact enediol system (13), the low titers indicate that at least some of the originally added ascorbic acid was derivatized at the 2-hydroxy or 3-hydroxy groups.

The yield of III in the final reaction mixtures could also be calculated from the titers of sodium hydroxide that were required to reach the phenolphthalein end-point. Details of this method were given previously (4). It depends on the fact that esterification of 1 mole of I by acetic anhydride produces 1 mole of acetic acid and 1 mole of ester, whereas hydrolysis of 1 mole of acetic anhydride yields 2 moles of acetic acid.

The experimental details for calculating yields of ester are given under *Experimental*, and a plot of yield of ester $[III]_e$ relative to the initial acetic anhydride $[Ac_2O]_0$ concentration against initial ascorbic acid concentration (at pH 6.0) is shown in Fig. 3. The plot in Fig. 3 reveals that the yield of ester relative to initial anhydride concentrations increases with increasing concentrations of I when these are low but becomes independent of the concentrations of I when these were above 1 × 10⁻¹ *M*. Furthermore, the maximum yield of ester never exceeded 87%. A possible explanation would be that at high concentrations of ascorbic acid relative to initial acetic anhydride concentrations, virtually all of the acetic anhydride reacted with I to yield II. It would then be necessary to conclude that II participated in two parallel reactions, rearrangement to III and hydrolysis, and that the ratio of the rate constants for these

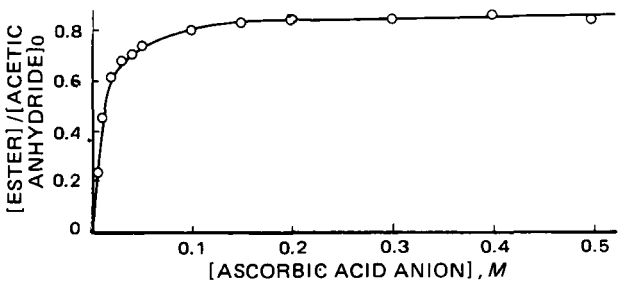


Figure 3—*Plot of yield of mono-O-acetyl ascorbate against initial concentration of sodium ascorbate ($[Ac_2O]_0$ = 0.02 M, pH 8.5, I = 1.5 M, T = 25°).*

reactions was insensitive to concentrations of I. This is considered to be the most likely explanation.

An alternative explanation for the results in Fig. 3 would be that ascorbate ion is catalyzing the hydrolysis of acetic anhydride by acting as a general base as well as by reacting to yield the ester, II. Although this possibility cannot be ruled out completely, evidence is presented subsequently to establish that hydrolysis and O → O acetyl transfer reactions both occur in II. Hence, any general base catalysis by ascorbate or hydrolysis of acetic anhydride will change the reaction sequence only slightly, and the paramount reactions appear to be those described. A strong suggestion that II would be likely to hydrolyze quite rapidly came from the observation that the closely related 5,6-isopropolidine-2,3-di-*O*-acetylascorbic acid hydrolyzed to the corresponding 2-*O*-acetyl derivative, with half-lives ($t_{1/2}$ values) of the order of 10 min in the 3.5–6.5 pH range.

The calculation of rate constants for the reactions in Scheme I is complicated by the fact that hydrolysis of acetic anhydride to acetic acid competes with the formation of I. Estimates of the rate constants for the various reactions were made from the results of experiments designed on the basis of the following considerations:

1. The rate law for the conversion of I and I′ (I + I′ = I_T) to II and II′ would be:

$$-\text{rate}_{[I]_T} = k_1^{obs}[Ac_2O][I]_T \qquad \text{(Eq. 3)}$$

2. Most of the acetic anhydride would be consumed by hydrolysis if the initial acetic anhydride concentration $[Ac_2O]_0$ was greatly in excess of the initial concentration of $[I]_T$. Hence, because the hydrolysis of acetic anhydride in aqueous buffers is a pseudo-first-order reaction:

$$[Ac_2O] = [Ac_2O]_0 e^{-k_{H_2O}^{obs}t} \qquad \text{(Eq. 4)}$$

The $k_{H_2O}^{obs}$ value in Eq. 4 is the pseudo-first-order rate constant for hydrolysis of acetic anhydride in a particular buffer solution at a particular pH value. If I and I′ do not significantly affect the rate of hydrolysis (*e.g.*, by acting as general acids or general bases), then the $k_{H_2O}^{obs}$ value in a reaction solution containing $[I]_T$ would be identical to that in the same solution without $[I]_T$. In experiments designed to study the rate of formation of II, the concentration of $[I]_T$ was approximately 5×10^{-5} *M*. It was assumed that these low concentrations would not significantly affect the value of $k_{H_2O}^{obs}$ and that the value of this latter parameter could be determined from experiments in which no ascorbic acid was included in the reaction solutions. Values of $k_{H_2O}^{obs}$ at several pH values are listed in Table I.

3. Substitution of Eq. 4 in Eq. 3 yields:

$$-\text{rate}_{[I]_T} = k_1^{obs}[Ac_2O]_0[I]_T e^{-k_{H_2O}^{obs}t} \qquad \text{(Eq. 5)}$$

4. If the conversion of I to II and the hydrolysis of acetic anhydride were the only reactions occurring, and if II and acetic anhydride did not absorb UV energy appreciably at a wavelength where I absorbed strongly, then the absorbance at that wavelength would vary with time in accordance with Eq. 6:

$$\log A = \log A_0 + \frac{k_1^{obs}[Ac_2O]_0}{2.303k_{H_2O}^{obs}}(1 - e^{-k_{H_2O}^{obs}t}) \qquad \text{(Eq. 6)}$$

Hence, plots of log *A* against $e^{-k_{H_2O}^{obs}t}$ should be linear with slopes equal to $(k_1^{obs}[Ac_2O]_0/2.303k_{H_2O}^{obs})$, and the slopes of these lines should be linearly related to $[Ac_2O]_0$ and should equal zero when $[Ac_2O]_0$ equals zero. This type of dependence of log *A* on the value of $e^{-k_{H_2O}^{obs}t}$ was observed during the first 1.0 min following the addition of acetic anhydride (initial concentration 0.5–2 × 10^{-2} *M*) to solutions of ascorbic acid (initial concentration ~5 × 10^{-5} *M*).

Plots of log *A* against $e^{-k_{H_2O}^{obs}t}$ yielded straight lines, and Fig. 4 shows a typical plot of the slopes of these lines against $[Ac_2O]_0$. Although acetic anhydride did absorb slightly at 275 nm, it was included in equal concentrations in both sample and reference cells. Hence, the changes in absorbance caused by the hydrolysis of acetic anhydride were minimized. The assumption that II did not absorb UV energy appreciably at 275 nm was justified on the basis that the absorptivity of 5,6-isopropolidine-2,3-di-*O*-acetylascorbic acid was very low at this wavelength (ϵ = <50). Both this latter compound and II do not have a free 3-hydroxy group. It is believed that the conjugated pathway between this group and the C-1 carbonyl group is the major chromophore in I and its derivatives.

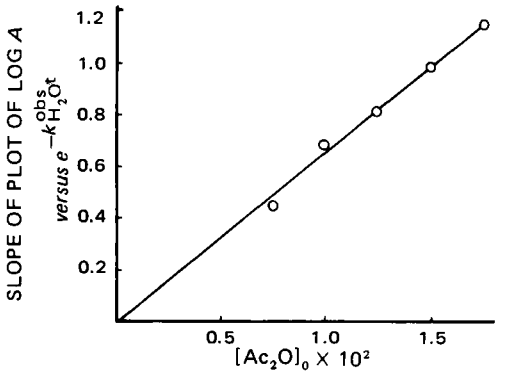


Figure 4—*Plot of slopes of plots of log* A versus $e^{-k_{H_2O}^{obs}t}$ *against initial acetic anhydride concentration (0.2* M *acetate buffer, pH 5.5, I = 1.0* M, T = 25°).

Kinetic evidence will be presented shortly to establish that very little conversion of II to III or back to I occurs during 1 min. Hence, it is believed that these experiments can be analyzed to obtain values of k_1^{obs}, as was done in the present study; values of k_1^{obs} are shown plotted against pH in Fig. 5. The solid line in this figure was calculated on the basis that the major reaction leading to formation of II involves acetic anhydride and the monoanion of I (*i.e.*, I^-). This finding is consistent with the postulate that the 3-hydroxy group of I is the first to be acylated in water at pH values between 2 and 6.

5. Because the reaction leading to the formation of II is a second-order reaction, its rate is very concentration dependent. On the other hand, the rearrangement and hydrolysis of II are postu-

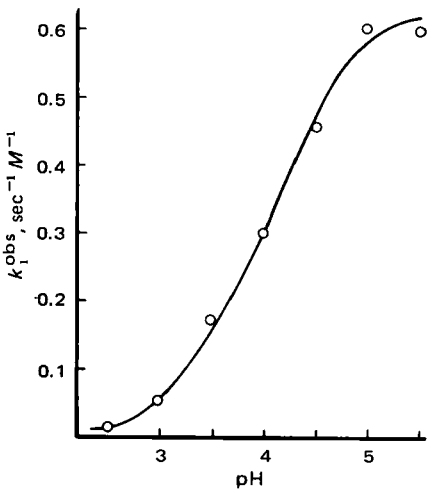


Figure 5—*The pH–rate profile of the second-order rate constants* (k_1^{obs}) *for the initial attack of acetic anhydride on ascorbic acid in a 0.2* M *acetate buffer (I = 1.0* M, T = 25°).

Table I—Rate Constants for Hydrolysis of Acetic Anhydride in 0.2 *M* Acetate Buffers at 25° (*I* = 1.0 *M*)

pH	$t_{1/2}$, min	$10^3 k_{H_2O}^{obs}$, sec^{-1}
2.5	4.55	2.49
3.5	5.12	2.24
4.0	4.80	2.33
4.5	4.73	2.44
5.0	4.26	2.71
5.5	3.72	3.04

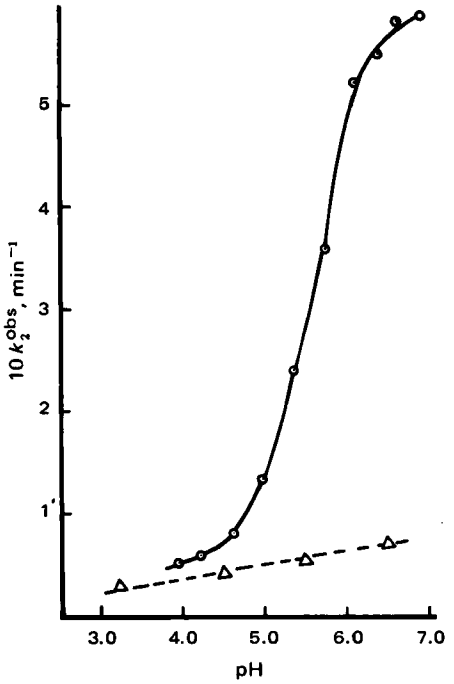


Figure 6—*The pH–rate profile of the overall rate of loss of 3-O-acetylascorbic acid (⊙) and rate of hydrolysis of 5,6-isopropolidine-2,3-di-O-acetylascorbic acid (△) (0.01 M citrate, I = 0.1 M, T = 25°).*

lated to be first-order (or pseudo-first-order) reactions in aqueous buffers, and their rates should be independent of the concentration of II. These postulates are the bases for the experiments employed to estimate the rate of conversion of II to III and I. A solution that was initially 10^{-2} *M* in both I and acetic anhydride and was buffered at pH 4.0 was prepared and kept at 25° for 4 min. The major reactions occurring under these conditions were the hydrolysis of acetic anhydride and the formation of II.

After 2 min, an aliquot of the solution was diluted ~200-fold into a buffer, and changes in UV absorbance at λ_{max} were recorded. Plots of log $(A - A_\infty)$ against time were linear for at least four half-lives, and these plots were used to calculate first-order rate constants, k^{obs} values, for the reactions that were occurring. A plot of k^{obs} values against the pH values of the buffers in which the reactions took place are displayed in Fig. 6.

Upon dilution of the original reaction mixture, any second-order reactions between acetic anhydride and $[I]_T$ (or possibly II or III) would be greatly slowed down. Hence, it is believed that the absorbance changes were caused by both the rearrangement of II to III (with a first-order rate constant k_2^{obs}) and hydrolysis of II (with a pseudo-first-order rate constant k_3^{obs}). On this basis, k^{obs} values would be related to the rate constants for the various reactions by:

$$k^{obs} = k_2^{obs} + k_3^{obs} \qquad \text{(Eq. 7)}$$

The fact that the pH profile in Fig. 6 has an inflection point at pH 5.5 suggests that the substrate has a pKa value of 5.5. This value is believed to be the pKa value of II. Although the second pKa value of I (*i.e.*, ionization of the 2-hydroxy group) is 11.5, the pKa value of II is expected to be much lower because it has an electron-withdrawing *O*-acetyl group adjacent to the ionizing group rather than a negatively charged oxygen atom.

The magnitudes of the rate constants k_2^{obs} and k_3^{obs} could not be calculated from values of k^{obs} alone. However, the fact that the yield of III is 87% at pH 6.0 suggests that the ratio k_2^{obs}/k_3^{obs} has a value of approximately 7 at this pH. Also, the observation that the UV spectra of final reaction mixtures were much closer to those of the initial solutions at pH <4 than at pH 4–6 suggests that the value of the ratio k_2^{obs}/k_3^{obs} is lower at pH 4 than it is at pH 6. Hence, it appears likely that the rearrangement reaction occurs most favorably in the ionized form of II (II′).

Although the ester III was much more stable than II, it slowly degraded in aqueous buffers. After standing at 25° for several days, solutions that originally contained III had identical UV spectra to solutions that contained equivalent amounts of I. Hence, it appears that III is slowly hydrolyzed ($t_{1/2} \simeq 24$ hr at pH 4.5) to ascorbic and acetic acids. Rate constants for the various reactions that were calculated from changes in optical rotation or by using a pH-stat technique were very close to those calculated from measurements of changes in UV absorbance.

No evidence was found for any migration of acyl groups from the 2-hydroxy or 3-hydroxy groups to the 5-hydroxy or 6-hydroxy groups as previously reported (1). These migrations would only be likely at pH values where the 5-hydroxy and 6-hydroxy groups are appreciably ionized. Because these hydroxy groups are so weakly acidic, much higher pH values than 7.0 would be required.

During this study, some questions arose about structural assignments that had been previously made to some ascorbic acid esters. Nomura and Sugimoto (1) synthesized a di-*O*-benzoylascorbic acid, which had a melting point within the 149–151° range and a pKa in methanol of 4.00. It was suggested that this ester was 3,6-di-*O*-benzoylascorbic acid. However, based on the finding in the present study that II has a pKa in water (which probably would be lower than that in methanol) of about 5.5, whereas III has a pKa in water of about 2, it seems likely that Nomura and Sugimoto's compound was the 2,6-di-*O*-benzoyl derivative.

Furthermore, it was reported by Nomura and Sugimoto (1) that 2,6-di-*O*-benzoylascorbic acid rearranged to the 3,6-isomer in base. Again, the present study leads to the conclusion that it was the 3,6-isomer that isomerized to yield the 2,6-isomer. Other workers (13) synthesized a di-*O*-benzoyl ester of ascorbic acid, which had a melting point of 151–152°, and assigned it the structure of 2,6-di-*O*-benzoylascorbic acid. This compound is probably the same one that Nomura and Sugimoto (1) synthesized.

Vestling and Rebstock (11) carried out an acetylation of 5,6-isopropolidineascorbic acid in acetone with ketene. These authors isolated a compound which melted at 113–115° and had the elemental analysis of: C, 51.44%; H, 5.55%. It was suggested that this compound was the 3-*O*-acetate of 5,6-isopropolidineascorbic acid. The theoretical values for the elemental analysis of this latter compound are: C, 51.2%; H, 5.42%. This synthesis was repeated in the present study and a compound was obtained with a melting point of 113–115° and a similar elemental analysis to that found by Vestling and Rebstock (11). However, this product is believed to be 5,6-isopropolidine-2,3-di-*O*-acetylascorbic acid. The reasons for reaching this conclusion are:

1. The theoretical elemental analysis figures for the diacetate are: C, 52.0%; H, 5.33%.
2. The mass spectrum of the compound had a peak at *m/e* 300, which is the molecular weight of the diacetate.
3. The NMR spectrum of the compound in $CDCl_3$ did not contain singlet proton peaks at or near δ 10.8 or 8.0 (which is where the 2-hydroxy and 3-hydroxy protons of I and 5,6-isopropolidineascorbic acid resonate), but it contained two peaks at δ 2.35, which integrated for six protons. These peaks would be caused by the six protons on the two acetyl peaks of 5,6-isopropolidine-2,3-di-*O*-acetylascorbic acid.

When this compound was dissolved in aqueous buffers with pH values between 2 and 7, the solution developed a UV spectrum very similar to that of III. Hence, it is believed that the diacetate hydrolyzes to 2-*O*-acetylisopropolidineascorbic acid, in agreement with the assignment of Sumiki *et al.* (5).

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Chemical and Pharmacological Investigations of Constituents of *Eleutherine bulbosa* (Miller) Urb. (Iridaceae)

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Abstract □ Eleutherin and eleutherol extracted from bulbs of *Eleutherine bulbosa* (Miller) Urb. (Iridaceae), collected in the Amazonian jungle and grown in Italy, were tested for biological properties. The extraction procedure and the results of antibacterial, cytotoxicity, and pharmacological assays are reported. Eleutherin has a weak and transient effect of decreasing the prothrombin time (*in vivo* in rats) and a weak antibacterial activity on *Bacillus subtilis (in vitro)*.

Keyphrases □ *Eleutherine bulbosa* (Iridaceae)—extraction of eleutherin and eleutherol, pharmacological screening □ Eleutherol and eleutherin—extracted from *Eleutherine bulbosa*, pharmacological screening □ Medicinal plants—extraction of eleutherin and eleutherol from *Eleutherine bulbosa*, pharmacological screening

In 1959, unknown bulbs, collected in the Peruvian part of the Amazonian jungle along the Ucayali River near Pucallpa[1], were sent to Europe[2]. Some bulbs, which arrived presumably a few months after collection, were planted in northern Italy near Milan at about 220 m above sea level, while others were subjected to biological screening.

Following the procedure described in this paper, a crystalline yellow substance was extracted from the dry bulbs. The substance proved to be a mixture of eleutherol and eleutherin[3]. The two compounds were separated, crystallized, and biologically assayed. Through specimens of bulbs and flowers obtained from the plant grown in Italy, the plant was identified[4] as *Eleutherine bulbosa* (Miller) Urb. [*E. plicata* (Ser.) Herb.].

Both eleutherol and eleutherin were described and chemically characterized earlier (1–3) by extracting the compounds from bulbs of *E. bulbosa* (Miller) Urb. (Iridaceae) collected in Java. The plant, cultivated in Java where it was used in a variety of illnesses by natives, originally came from equatorial America (1). A related plant, *E. plicata*, is said to be a popular medicine used by natives of north or northeast Brazil. Some studies on the chemical constituents of the plant have been performed (4).

While data on the chemical properties of eleutherol and eleutherin are abundant (1–3, 5–14), data on their biological properties are scarce (6). This paper reports the results of experiments performed on a sample of eleutherol and eleutherin extracted from bulbs of *E. bulbosa* grown in Italy.

EXPERIMENTAL[5]

Bulbs grown in Amazonia were used in the early phases of the research, while bulbs grown in Italy were used subsequently. The only apparent difference between them was that the yellow crystalline material on the outer scales of dry bulbs was more abundant on those of Amazonian origin than on those of Italian origin. The bulbs planted in Italy were allowed to grow for about 6–7 months; they then were harvested, separated from the aboveground parts, and air dried for about 3 months at room temperature.

Dry bulbs (1 kg) were sliced and left to macerate with 1 liter of 95% ethanol for 2 days; the procedure was repeated once. The filtered alcoholic extracts were combined and brought to dryness at 50° under reduced pressure. The residue was dissolved in warm acetone, and one volume of ether and six volumes of water were added to it. The aqueous phase was separated and extracted four times with a total amount of ether corresponding to 1.5–2 times the volume of the water. All ether extracts were combined, dried over anhydrous sodium sulfate, filtered, and brought to dryness under reduced pressure.

The residue was dissolved in a minimum amount of warm absolute ethanol. Refrigeration of the ethanol solution yielded a yellow crystalline material, which was recrystallized once from absolute

[1] For details about the geographical position of Pucallpa and for a view of the jungle along the Ucayali River, see *National Geographic*, Feb. 1964, and the companion map.

[2] People living in Pucallpa indicated that the aborigines call the plant "Piri-Piri" and use decoctions of the bulbs to stop postpartum hemorrhages.

[3] Identification was carried out by Professor A. Quilico, Istituto di Chimica Generale e Analitica, Politecnico di Milano, Milan, Italy.

[4] Identification was carried out by the late Professor F. Morton of Hallstatt. The specimen is deposited at the Botanischer Garten und Botanisches Museum, Berlin, Dahlem, Germany (herb. D. E. Meyer No. 2286).

[5] Melting points were taken on a Leitz hot-stage microscope and are uncorrected. Optical rotation measurements were determined on a Perkin-Elmer 141 polarimeter. UV spectra were determined in ethanol using a Beckman DU spectrophotometer. TLC was carried out on silica gel G (0.25 mm) plates using benzene–acetone (9:1) (Solvent A) and petroleum ether–ethyl acetate–chloroform (67:33:10) (Solvent B) as developing systems. Separated components on the plates were visualized by their appearance under 254-nm UV light. For TLC, 10 or 20 μg of the material under test was applied.