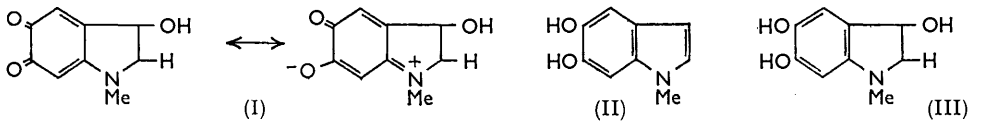
879. *The Mechanism of the Reduction of Adrenochrome by Ascorbic Acid*

By G. L. Mattok

The mechanism of the reduction of adrenochrome by ascorbic acid in water and methanol has been investigated. The rate of reduction is directly dependent on the concentration of adrenochrome; rates of reduction in methanol are also first-order with respect to the ascorbic acid concentration. At the lowest concentration of ascorbic acid used the reductions in methanol are more rapid than similar reductions in water using the same total ascorbic acid concentration. However, reactions in water do show a first-order dependence on the concentration of the fraction of undissociated ascorbic acid present. Addition of dehydroascorbic acid to these reactions in methanol decreases the rate of reduction while there is an increase when dehydroascorbic acid is added to the reductions in water. A mechanism for the reduction of adrenochrome by ascorbic acid is proposed.

The products obtained when adrenochrome * (I) is reduced by ascorbic acid have been established as 5,6-dihydroxy-*N*-methylindole (II) [2] and a secondary product [3,4] resulting from an interaction of 5,6-dihydroxy-*N*-methylindole and the dehydroascorbic acid formed from the oxidation of ascorbic acid; the primary reduction product from adrenochrome is probably " leucoadrenochrome " (III) which rapidly undergoes dehydration to form



5,6-dihydroxy-*N*-methylindole (II). Although several other studies of similar systems have been reported [5-7] only one of these [5] considered the mechanism of the reaction. Roston [5] suggested that the reduction of oxidised noradrenaline by ascorbic acid required a large excess of ascorbic acid and, therefore, likely mechanisms would include a reaction which is first-order with respect to aminochrome concentration or " a rearrangement in an environment modified by ascorbic acid." Further, Roston found that the oxidised noradrenaline disappeared relatively more rapidly as its concentration was increased and the process was, therefore, not first-order with respect to the concentration of this reagent; Roston's results also showed that the rate of this reduction was strongly dependent on the level of oxygen in the solutions and he, therefore, considered that " the ascorbic acid appeared to modify the environment through reduction in free oxygen." However, Roston's results were obtained with solution of oxidised catecholamines, prepared by bubbling oxygen through a solution of the catecholamine in hydrochloric acid, in the presence of manganese dioxide. Studies of the reduction of noradrenochrome, prepared in this way, by ascorbic acid are open to two sources of error: (*a*) pure, solid noradrenochrome has not yet been isolated and it is difficult to prepare solutions [8] of it without contamination by

* The 2,3-dihydroindole-5,6-quinones (obtained by the oxidative cyclisation of catecholamines) are known as the " aminochromes." The physical and chemical properties [1] of adrenochrome, which is a typical aminochrome, suggest that the zwitterionic form of the molecule makes the major contribution to the adrenochrome structure.

[1] J. Harley-Mason, *Experientia*, 1948, **4**, 307.
[2] R. A. Heacock and B. D. Laidlaw, *Nature*, 1958, **182**, 526.
[3] G. L. Mattok and R. A. Heacock, *Nature*, 1963, **198**, 993.
[4] G. L. Mattok and R. A. Heacock, *Canad. J. Chem.*, 1964, **42**, 1401.
[5] S. Roston, *Nature*, 1962, **194**, 1079.
[6] A. Beauvillain and J. Sarradin, *Bull. Soc. Chim. biol.*, 1948, **30**, 478.
[7] P. Fischer, *Bull. Soc. chim. belges*, 1949, **58**, 205.
[8] R. A. Heacock and G. L. Mattok, *Canad. J. Chem.*, 1963, **41**, 139.

partly oxidised catecholamine and other substances; (*b*) the rate of autoxidation of ascorbic acid is markedly enhanced by the presence of traces of the ions of heavy metals.[9] When manganese dioxide is used in the oxidation of the catecholamine it is probable that the oxidised solution contains traces of ions of manganese, which would catalyse the autoxidation of ascorbic acid; it might then appear that relatively large amounts of ascorbic acid are required to reduce the aminochrome.

Several recent publications have described, from the kinetic standpoint, other reductions by ascorbic acid. Some of these investigations have been directed towards an understanding of the model hydroxylating system [10] consisting of ascorbic acid, the ferric chelate of ethylenediaminetetra-acetic acid, and oxygen. Grinstead [11] has shown that the oxidation of ascorbic acid by the ferric chelate of ethylenediaminetetra-acetic acid involves a rate-determining one-electron oxidation of the ascorbic acid to the ascorbate anion-radical; in the presence of hydrogen peroxide the oxidation involves a chain process initiated by the above reduction step. The ascorbate anion-radical is also thought to be involved in the hydroxylation of quinoline [12] by the ferrous sulphate–ethylenediaminetetra-acetic acid–ascorbic acid–hydrogen peroxide or oxygen model hydroxylating systems. Yamazaki, Mason, and Piette [13] have detected, by electron paramagnetic resonance spectroscopy, a free radical generated during the oxidation, at pH 4·8, of ascorbic acid by hydrogen peroxide. A free-radical mechanism [14] has also been proposed for the ascorbic acid oxidase and peroxidase reactions.

Results

Order of the Reaction.—The relationship between the absorbance of the reaction mixture and time was approximately linear for all the mixtures and the initial rates could, therefore, be determined accurately (±5%), using the plane-mirror technique; in view of the changing concentrations of undissociated and dissociated ascorbic acid owing to shifts in the ascorbic acid–ascorbate equilibrium (see below) as the reactions in water proceed, the method of initial slopes is well suited for order determinations in this case. The order of the reaction with respect to the concentration of each reactant was determined from the effect of varying, in turn, the concentrations of adrenochrome ($1{\cdot}47 \times 10^{-4}$M—$4{\cdot}44 \times 10^{-4}$M) and ascorbic acid ($0{\cdot}98 \times 10^{-4}$—$5{\cdot}88 \times 10^{-4}$M) in the reaction mixtures on the initial rates of the reactions. The rate of reduction of adrenochrome by ascorbic acid, in water or methanol, is directly proportional to the adrenochrome concentration (Tables 1 and 2). In methanol the rate of reduction also shows first-order dependency on the ascorbic acid concentration; calculations of rate constants at several times during the course of the reaction confirm that the reduction, in methanol, obeys overall second-order kinetics (Table 3). When the method of initial slopes is used to determine the order of the reduction in water, the reduction appears to be approximately second-order with respect to the concentration of ascorbic acid. Analysis of individual runs in water, using the integrated rate expressions, did not give constant values of either second- or third-order rate constants; however, there is a first-order dependence of the initial rates on the concentrations of undissociated ascorbic acid calculated from the pH of the ascorbic acid solutions and a literature value [15] of the pK_a (4·12) for ascorbic acid (Table 1).

Effect of Dehydroascorbic Acid on the Rate of Reduction.—When dehydroascorbic acid is added to adrenochrome–ascorbic acid reaction mixtures in water the rate of reduction increases with increased concentration of dehydroascorbic acid; at high levels of dehydroascorbic acid the rates become fairly constant, and close to that found for reductions in methanol with similar ascorbic acid concentrations and no dehydroascorbic acid (Figure 1). This increased activity of the ascorbic acid–dehydroascorbic acid system is not, due to the breakdown of the dehydroascorbic acid to 2,3-dioxogulonic acid and other substances with strongly reducing properties since adrenochrome, in aqueous solutions containing comparable amounts of

[9] A. E. Kellie and S. S. Zilva, *Biochem. J.*, 1935, **29**, 1028.
[10] S. Udenfriend, C. T. Clark, J. Axelrod, and B. B. Brodie, *J. Biol. Chem.*, 1954, **208**, 731.
[11] R. R. Grinstead, *J. Amer. Chem. Soc.*, 1960, **82**, 3464, 3472.
[12] R. Breslow and L. N. Lukens, *J. Biol. Chem.*, 1960, **235**, 292.
[13] I. Yamazaki, H. S. Mason, and L. Piette, *J. Biol. Chem.*, 1960, **235**, 2444.
[14] I. Yamazaki and L. Piette, *Biochem. Biophys. Acta*, 1961, **50**, 62.
[15] J. Ball, *J. Biol. Chem.*, 1937, **118**, 219.

TABLE 1

Reduction of adrenochrome by ascorbic acid in water at 32·8°: order of the reaction

Adrenochrome (10^{-4}M)	Total ascorbic acid (10^{-4}M)	pH	Undissociated * ascorbic acid (10^{-4}M)	Initial rate (10^{-6} mole l.$^{-1}$ min.$^{-1}$)	$10^{-2}k_2$ (l. mole^{-1} min.$^{-1}$)
1·47	3·92	4·25	1·68	4·54	1·85
1·96	3·92	4·25	1·68	6·07	1·85
2·94	3·92	4·25	1·68	9·22	1·87
3·92	3·92	4·25	1·68	12·45	1·90
4·44	3·92	4·25	1·68	13·90	1·87
1·96	0·98	5·55	0·04	0·16	2·05
1·96	1·96	4·70	0·43	1·47	1·84
1·96	2·94	4·40	1·01	3·88	1·96
1·96	4·90	4·15	2·37	9·55	2·05
1·96	5·88	4·10	3·02	12·22	2·06

* Calculated from $pK_a = 4·12$.

TABLE 2

Reduction of adrenochrome by ascorbic acid in methanol at 32·8°

(*a*) *Dependence of the initial rate on adrenochrome concentration* [Ascorbic acid] = $3·92 \times 10^{-4}$M

Adrenochrome (10^{-4}M)	0·98	1·47	1·96	2·94	3·92
Initial rate (10^{-6} mole l.$^{-1}$ min.$^{-1}$)	4·47	7·20	8·75	13·7	20·0
$10^{-2}k_2$ (l. mole^{-1} min.$^{-1}$)	1·16	1·25	1·14	1·19	1·30

(*b*) *Dependence of the initial rate on ascorbic acid concentration* [Adrenochrome] = $1·96 \times 10^{-4}$M

Ascorbic acid (10^{-4}M)	0·98	1·96	2·94	3·92	4·90	5·88
Initial rate (10^{-6} mole l.$^{-1}$ min.$^{-1}$)	2·35	4·37	6·75	9·00	12·9	14·1
$10^{-2}k_2$ (l. mole^{-1} min.$^{-1}$)	1·23	1·14	1·18	1·18	1·34	1·22

TABLE 3

Reduction of adrenochrome by ascorbic acid in methanol at 32·8°: typical analyses for the order of the reaction

[Adrenochrome] = $1·96 \times 10^{-4}$M; [Ascorbic acid] = $1·96 \times 10^{-4}$M

Time (min.)	A * ($\times 10^2$)	k_2 (l. mole^{-1} min.$^{-1}$)	$10^{-4}k_3$ (l.^{2} mole^{-2} min.$^{-1}$)	Time (min.)	A_t * ($\times 10^2$)	k_2 (l. mole^{-1} min.$^{-1}$)	$10^{-4}k_3$ (l.^{2} mole^{-2} min.$^{-1}$)
0	72·0	—	—	9	60·0	111	80
1	70·5	107	55	11	58·1	109	85
2	69·0	108	62	15	54·5	107	97
3	67·3	117	66	18	52·0	109	111
4	66·0	114	68	20	50·4	109	126
5	64·8	111	69	25	46·7	111	165
7	62·6	107	71	30	44·2	106	208

* Absorbance at 472 mμ.

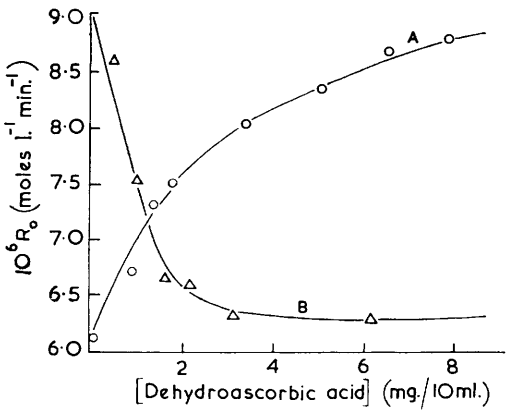


FIGURE 1. The reduction of adrenochrome ($1·96 \times 10^{-4}$M) by ascorbic acid ($3·92 \times 10^{-4}$M) in the presence of dehydroascorbic acid (methanol complex) in (A) water and (B) methanol, at 32·8°. Dependence of the initial rate of reduction (R_0) on the concentration of dehydroascorbic acid

dehydroascorbic acid, was stable for several hours. When dehydroascorbic acid is added to the adrenochrome–ascorbic acid reaction mixtures in methanol, there is a decrease in the rate of reduction of adrenochrome; the rate of reduction decreases with increased dehydroascorbic acid concentration and finally becomes constant at high dehydroascorbic acid levels. This is in direct contrast with the observation for reductions in water (Figure 1).

Effect of pH *on the Rate of Reduction.*—Sodium ascorbate in aqueous solution does not reduce adrenochrome, but rearrangement to a fluorescent product, adrenolutin (*i.e.*, 5,6-dihydroxy-*N*-methylindoxyl) occurs. A study of the effect of pH, using a series of McIlvaine (Na_2HPO_4–citric acid) buffer solutions, on the rate of reduction of adrenochrome was limited to the pH range 2·5—5·0, since adrenochrome, in these solutions, is relatively unstable outside these limits. The rate of reduction of adrenochrome by ascorbic acid increases as the pH of the reaction medium is decreased and shows a first-order dependence on the concentration of undissociated ascorbic acid calculated using the literature value [15] of $pK_a = 4·12$ (Table 4).

TABLE 4

Reduction of adrenochrome by ascorbic acid in McIlvaine buffer solutions at 32·8°: dependence of the initial rate (R_0) on the concentration of undissociated ascorbic acid (AH_2).

Initial concentrations: [adrenochrome] = $1·56 \times 10^{-4}$M; [ascorbic acid] = $1·74 \times 10^{-4}$M

pH	2·5	3·1	3·6	4·1	4·5	5·0
AH_2 (10^{-4}M)	1·70	1·58	1·32	0·87	0·50	0·19
10^6R_0 (mole $l.^{-1}$ $min.^{-1}$)	5·74	5·01	4·45	2·75	1·56	0·73

Possible Ascorbic Acid Complexes.—The possibility of association between (*a*) ascorbic acid and dehydroascorbic acid in methanol and (*b*) ascorbic acid and ascorbate ion in water, was investigated by osmometric measurements of vapour pressure equilibria in these solutions. The results are summarised in Table 5. In the case of the ascorbic acid–dehydroascorbic acid solution in methanol the change in resistance (ΔR) calculated from measurements on solutions of the components, is slightly higher than the ΔR value found for the mixture. There is, therefore, no association between ascorbic acid and dehydroascorbic acid in methanol although the high calculated value indicates that some dissociation of one of the components of the mixture may have occurred; however, evidence has been reported [16] which suggests that association between ascorbic acid and dehydroascorbic acid does occur in water. The value of ΔR obtained for the ascorbic acid–ascorbate solution in water is strictly additive with respect to the ΔR values obtained for solutions of each component; no association, therefore, takes place between ascorbic acid and ascorbate ion, although it has been proposed that a salt–acid complex is formed during the anærobic degradation of ascorbic acid in aqueous solution.[17]

TABLE 5

Vapour pressure equilibria for solutions of ascorbic acid and (*a*) dehydroascorbic acid in methanol, (*a*) sodium ascorbate in water. Changes in resistance (ΔR) at 37°

In methanol (1 ml.)		*In water* (1 ml.)	
Solute *	ΔR	Solute *	ΔR
(I) AA (16·516 mg.)	18·90	(IV) AA (16·960 mg.)	5·45
(II) DHA (15·973)	10·88	(V) NaAA (10·745 mg.)	5·99
(III) AA (8·466 mg.) + DHA (6·177 mg.)	14·70	(VI) AA (17·563 mg.) + NaAA (10·425 mg.)	11·54
(III) Calc. (for 1 : 1 complex)	9·62	(VI) Calc. (for 1 : 1 complex)	8·51
(III) Calc. (if no association)	13·80	(VI) Calc. (if no association)	11·45

* AA = ascorbic acid; DHA = dehydroascorbic acid (methanol complex); NaAA = sodium ascorbate.

Ultraviolet Spectra of Ascorbic Acid Solutions.—The conjugated enediol group of ascorbic acid gives an intense absorption band (ε 8800) at 245 mμ in acid solution, which moves to 265 mμ (ε 11,100) in neutral solution following dissociation [18] of the enol group on carbon-3; at the highest concentration of ascorbic acid ($1·4 \times 10^{-4}$M) for which absorbance measurements in the ultraviolet region are possible, the maximum appears at 260 mμ, corresponding to the

[16] N. I. Gryaznov and V. K. Kunschikova, *Voprosy Pitaniya*, 1940, **9**, 84 (*Chem. Abs.*, 1943, **37**, 5699).

[17] P. Finholt, R. B. Paulssen, and T. Higuchi, *J. Pharm. Sci.*, 1963, **52**, 948.

[18] R. W. Herbert, E. L. Hirst, E. G. V. Percival, R. J. W. Reynolds, and F. Smith, *J.*, 1933, 1270.

presence of some undissociated acid. When dehydroascorbic acid [which is transparent in the region 230—280 mμ and shows a weak absorption band (ε 720) at 300 mμ] is added to dilute aqueous solutions of ascorbic acid the maximum shifts to 245 mμ (Figure 2) corresponding to an increase in the concentration of the undissociated acid ($\lambda_{max.}$ 245 mμ).

Solutions of ascorbic acid in methanol, at concentrations greater than $1{\cdot}2 \times 10^{-4}$M, show a maximum at 245 mμ. Dilution of these solutions results in a shift towards 265 mμ owing to increased dissociation of the ascorbic acid; when the concentration is $0{\cdot}6 \times 10^{-4}$M, the maximum absorption band is at 265 mμ. Addition of dehydroascorbic acid to methanolic solutions of ascorbic acid results in changes in the ultraviolet spectra which are similar to those observed for aqueous solutions; all solutions of ascorbic acid in methanol containing dehydroascorbic acid possessed maxima at 245 mμ (Figure 2).

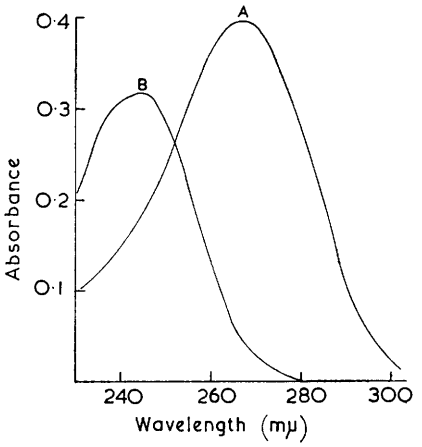


FIGURE 2. (A) Absorption spectrum of ascorbic acid ($3{\cdot}5 \times 10^{-5}$M) in water and (B) resultant spectrum after addition of dehydroascorbic acid methanol complex (0·1 mg./ml.) to A

Electron Paramagnetic Resonance Spectra.—Spectra were obtained during the reduction of adrenochrome (5×10^{-2}M) by ascorbic acid (5×10^{-2}M) in water and methanol using transfer or flow techniques. The free-radical signal obtained was very weak with a *g*-value close to that of diphenylpicrylhydrazyl (DPPH), *i.e.*, 2·0036, and with a band width of the order of 12 gauss; however, it was necessary to use high modulation to obtain the spectrum and this would result in a broadening of the signal. The free-radical signal obtained by Yamazaki, Mason, and Piette,[13] during the oxidation of ascorbic acid by peroxidase, was a doublet; the centre of the low-field line was at $g = 2{\cdot}0043$ and the total width of the signal was *ca.* 12 gauss.

DISCUSSION

The rate of reduction of adrenochrome by ascorbic acid in methanol is directly dependent on the concentrations of adrenochrome and ascorbic acid and calculation of second- and third-order rate constants at several times during the course of individual runs confirms that the reduction obeys overall second-order kinetics (Table 3). The ultraviolet spectra of solutions of ascorbic acid in methanol show that there is very little dissociation of ascorbic acid in this solvent over the range of concentrations used.

Rates of reactions in water are directly dependent on the adrenochrome concentrations; there is no simple dependence of the initial rates on the initial total ascorbic acid concentrations and a six-fold decrease in the aqueous ascorbic acid concentration results in an eighty-fold decrease in the rate of reduction (see Table 1). Second- and third-order rate constants, calculated at several times during the course of individual runs, varied by approximately the same extent and, therefore, did not provide a basis for the determination of the overall order of the reduction in water. However, there is a good first-order dependence of the initial rates on the calculated initial concentrations of undissociated ascorbic acid (Table 1) and the variation of the second-order rate constant with reaction time is, therefore, due to the continuously shifting ascorbic acid–ascorbate equilibrium as the reaction proceeds. The importance of the undissociated ascorbic acid in the reduction of adrenochrome by ascorbic acid in water, as in methanol, is confirmed by the first-order

dependence of the rate of reduction on the calculated concentrations of this fraction when the reductions were carried out in a series of buffer solutions (Table 4). These results suggest that reduction by the ascorbate ion is negligible compared with the main reduction by the undissociated ascorbic acid. Sodium ascorbate in aqueous solution (pH 7·2) does not reduce adrenochrome; however, this may be due to the necessity for an initial rapid protonation of the polarised C-6 carbonyl group in adrenochrome. Aqueous solutions of adrenochrome show an absorption maximum, in the visible region, at *ca.* 485 mμ; when hydrochloric acid is added to these solutions the absorbance at this wavelength is reduced and a new maximum appears at *ca.* 400 mμ, due to protonation of the C-6 carbonyl group.[19] These changes are not observed when ascorbic acid is added to aqueous solutions of adrenochrome. A further indication that this initial rapid protonation does not take place is provided by the fact that rates of reduction, using methanol as solvent, are greater than those obtained for similar reductions in water (Tables 1 and 2) despite the virtual absence of dissociation of ascorbic acid in methanol and the lower proportion of the zwitterionic form of adrenochrome, as suggested by the shift in $\lambda_{max.}$ for these solutions from 487 mμ in water to 472 mμ in methanol.

The mechanisms of reductions by ascorbic acid are generally thought to involve a free radical derived from ascorbic acid.[11-14] In view of the fact that the reduction of adrenochrome by this reagent takes place in methanol as well as, or better than, in water an ionic mechanism does not seem likely. E.p.r. spectra of these reaction mixtures in water showed a weak signal in a similar position to and of comparable width with that reported by Yamazaki, Mason, and Piette [13] for the free radical formed during the autoxidation of ascorbic acid in the presence of hydrogen peroxide. These authors preferred a radical of the ascorbate ion, although the spectra were recorded at pH 4·8 where the ascorbic acid–ascorbate ion ratio is only 1 : 4. Since the rate of reduction of adrenochrome by ascorbic acid is dependent on the concentration of undissociated ascorbic acid, a radical derived from the enediol form is more likely in this case. The kinetics of the reduction of adrenochrome by ascorbic acid in water and methanol are explained satisfactorily by the following equations:

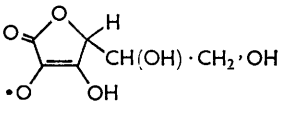
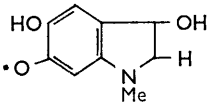
$$AH_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A\bar{H} + H \qquad (1)$$

$$AQ + AH_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} AQH\cdot + AH\cdot \text{ (slow)} \qquad (2)$$

$$AQH\cdot + AH_2 \xrightarrow{k_3} AQH_2 + AH\cdot \qquad (3)$$

$$2AH\cdot \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} A + AH_2 \qquad (4)$$

In this sequence AQ, AQH_2, AH_2, and A denote adrenochrome (I), leucoadrenochrome (III), ascorbic acid, and dehydroascorbic acid, respectively; AH· denotes the partially oxidised ascorbic acid free-radical intermediate (IV) and AQH· the partially reduced adrenochrome free-radical intermediate (V). Leucoadrenochrome (AQH_2) rapidly loses

(IV)   (V)

water [20] to form 5,6-dihydroxy-*N*-methylindole (II) and reaction (3) is, therefore, assumed irreversible. Assuming $k_2 \ll k_4$, equations (2), (3), and (4) give rise to the rate equation

$$\frac{d[AQH_2]}{dt} = \frac{k_2 k_3 [AQ][AH_2]^2}{k_{-2}\{k_{-4}[A][AH_2]/k_4\}^{\frac{1}{2}} + k_3[AH_2]} \qquad (i)$$

[19] J. D. Bu'Lock, *J.*, 1961, 52.
[20] J. Harley-Mason, *J.*, 1950, 1276.

Under normal reaction conditions, *i.e.*, when dehydroascorbic acid is not added to the reaction mixture, dehydroascorbic acid and 5,6-dihydroxy-*N*-methylindole react to form a 1,4-benzodioxan derivative [4] and [A] is then zero; equation (i) then becomes $d[AQH_2]/dt = k_2[AQ][AH_2]$ which is consistent with the observed kinetics for reductions in methanol where equilibrium (1) lies entirely to the left-hand side and $[AH_2]$ is equal to the total ascorbic concentration. In the case of reactions in water, the concentration of undissociated ascorbic acid is determined by the equilibrium (1), *i.e.*,

$$k = k_1/k_{-1} = [A\bar{H}][H^+]/[AH_2]$$

and the fraction of the undissociated acid varies widely (3·6% for a 0·98M-solution to 51% for a 5·88M-solution) over the range of concentrations used. Therefore, a first-order relationship between the rate of reduction and the concentration of undissociated acid in water, rather than the total ascorbic acid concentration, would be expected; this was, in fact, observed for reactions in water (Table 1) and in a series of buffer solutions (Table 4). A comparable effect is found when the rate constants are calculated at several times during the reduction when, after 10—15% reaction, the second-order rate constant begins to decrease, owing to a shift in the ascorbic acid–ascorbate equilibrium in favour of the ascorbate ion, as a result of the effective dilution of the ascorbic acid as it reacts.

The decrease in the rate of reduction of adrenochrome by ascorbic acid which results from the addition of dehydroascorbic acid to the reaction mixtures in methanol would be expected from equation (i). The presence of relatively high concentrations of dehydroascorbic acid would shift equilibrium (4) to the left, thereby reducing the amount of ascorbic acid available for reduction, and the resulting accumulation of ascorbic acid free radicals would, in turn, adversely affect product (AQH_2) formation by displacing equilibrium (2) towards the left-hand side. The decreased rates of reduction of adrenochrome in methanol, in the presence of dehydroascorbic acid, could result from a decrease in the effective concentration of undissociated ascorbic acid by some form of association with dehydroascorbic acid. Although the ultraviolet spectra of methanolic solutions of ascorbic acid are modified by the addition of dehydroascorbic acid (cf. Figure 2), the resultant spectra correspond in intensity and position to those expected for solutions containing equivalent amounts of undissociated ascorbic acid, and, therefore, association between ascorbic acid and dehydroascorbic acid in this solvent does not seem likely; furthermore, osmometric measurements with methanolic solutions containing dehydroascorbic and ascorbic acids gave results which were close to those calculated assuming no association between the solutes (Table 5). Association between ascorbic acid and dehydroascorbic acid does not, therefore, contribute to the decrease in the rates of reduction of adrenochrome in methanol.

Addition of dehydroascorbic acid to adrenochrome–ascorbic acid reaction mixtures, in water, resulted in an increase in the rate of reduction of adrenochrome, although a decrease would be expected from equation (i). The spectral changes observed when dehydroascorbic acid was added to aqueous solutions of ascorbic acid corresponded to an increase in the concentration of the undissociated ascorbic acid (Figure 2); the mechanism by which this occurs is not clear. However, these spectral changes could also be interpreted on the basis of interaction between the solutes, resulting in the formation of a monodehydroascorbic acid–ascorbic acid complex similar to that described by Levandoski, Baker, and Canham [21] as an intermediate in the oxidation of ascorbic acid to dehydroascorbic acid. This complex is reported to have an ultraviolet spectrum close to that of ascorbic acid and to catalyse the oxidation of ascorbic acid to 2,3-dioxogulonic acid. If the changes in the ultraviolet spectra of solutions of ascorbic acid which result from the addition of dehydroascorbic acid are due to the formation of a monodehydroascorbic acid–ascorbic acid complex, then, since the spectral changes for solutions of ascorbic acid in methanol and water are similar, it would be expected that the effect of the rates of reduction in these solvents

[21] N. G. Levandoski, E. M. Baker, and J. E. Canham, *Biochemistry*, 1964, **3**, 1465.

would also be similar; in fact, addition of dehydroascorbic acid to reactions in methanol results in decreased rates while in water the rates are increased (Figure 1). The enhanced rate of reduction of adrenochrome in aqueous solution containing dehydroascorbic acid is, therefore, probably due to an increased concentration of undissociated ascorbic acid and, presumably, the competing effect of the added dehydroascorbic acid, which reduces the rates owing to the increased concentration of ascorbic acid free radicals, is small, *i.e.*, $k_4 \gg k_{-4}$ in water; this is consistent with the observation [13,14] that no free-radical signal was detected from an aqueous solution (pH 4·8) of a mixture of ascorbic and dehydroascorbic acids.

Experimental

Materials.—Adrenochrome was prepared by the method of Heacock, Nerenberg, and Payza.[22] Ascorbic acid was obtained from B.D.H. Ltd. and dehydroascorbic acid (as its methanol complex) from the Nutritional Biochemicals Corporation. The methanol used was B.D.H. AnalaR grade. No precautions were taken to remove air from the water and methanol solvents since runs, using different batches of these solvents, reproduced well; this is in contrast with the observations of Roston [5] who found that the rate of disappearance of adrenochrome in the presence of ascorbic acid was slower when oxygen was removed from the aqueous solvent. Ascorbic acid is relatively stable in aqueous or methanolic solutions; less than 4% decrease in the absorbance at the $\lambda_{max.}$ of solutions in these solvents was observed after they had been allowed to stand for 2 hr. Kellie and Zilva [9] reported that ascorbic acid is quite stable when metallic salts are absent from its solutions and that there was no significant increase in the rate of disappearance of dissolved ascorbic acid when the water used as solvent had been previously saturated with oxygen; however, the rate of oxidation of ascorbic acid was greatly increased by the presence of traces of ions of heavy metals.

Kinetic Measurements.—The reduction of adrenochrome by ascorbic acid was studied in (*a*) water and (*b*) methanol. The progress of each reaction was followed by measuring the decrease in absorbance at the $\lambda_{max.}$ for adrenochrome (487 mμ in water and 472 mμ in methanol) with time, using a Beckman DK-2 spectrophotometer equipped with a temperature-regulated cell holder. The initial concentrations of the reagents are shown in Table 1.

Vapour Pressure Equilibria.—Measurements were made with a thermoelectric " osmometer " (Mechrolab Inc., Mountain View, California) using the appropriate probe at 37°. Changes in resistance (ΔR) of solutions of (*a*) ascorbic acid and dehydroascorbic acid in methanol and (*b*) ascorbic acid and sodium ascorbate in water were compared with values of ΔR obtained for solutions of the components alone. Values of ΔR were noted at 3 min. equilibration time. The results are summarised in Table 5.

Electron Paramagnetic Resonance Spectra.—The e.p.r. spectra were measured in the Varian flat-walled quartz aqueous-solution cell of a Varian V-4500 x-band spectrometer, utilising 100 kc./sec. field modulation. In the flow technique, the cell was first filled with the aqueous adrenochrome solution and the ascorbic acid solution added, from a pipette, at the same rate as the reaction mixture dripped from the lower outlet of the cell; when the concentrations of ascorbic acid and adrenochrome in the reaction mixture were estimated to be approximately equal, small volumes of solutions of each reactant were added in turn.

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[22] R. A. Heacock, C. Nerenberg, and A. N. Payza, *Canad. J. Chem.*, 1958, **36**, 853.