A Critical Examination of Lugg's Method for the Determination of *l*-Ascorbic Acid

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Recently Lugg [1942 *a, b*] has described a procedure designed to increase the specificity of the determination of *l*-ascorbic acid by indophenol titration. The method is based on the differential rates of combination of *l*-ascorbic acid and some other indophenol-reducing substances with HCHO at pH 1.5 and at 3.5. After reaction with HCHO these substances no longer reduce the indicator. In order to ascertain the limitations of this method, Lugg's results have been submitted to a critical examination. In addition we record very briefly some experiments from an investigation which was in progress when Lugg's paper appeared. These were also intended to serve as a basis for a more specific determination of *l*-ascorbic acid and were concerned with the differential properties of the primary oxidation products of *l*-ascorbic acid, reductone and reductic acid. Although the results obtained were less satisfactory than Lugg's, they were not without certain interest.

METHODS

In our investigation we have introduced a few minor modifications which in no way interfered with the general principle enunciated by Lugg. Instead of metaphosphoric acid we employed trichloroacetic acid (final concentration in extract 2.5%). As all our experiments were performed on solutions of pure compounds, the buffers employed were chosen to represent the concentrations of trichloroacetate and citrate which would be present in an extract adjusted to the requisite pH in the course of a determination by the HCHO technique. Also, in carrying out the indophenol titrations, we did not find it necessary to use the 'drop persistence' technique of Lugg. After the direct addition of the indicator the usual correction was made for the amount of dye required to produce the tint observed at the end-point.

Reagents

Formaldehyde—the solutions of formaldehyde were prepared from the Analar reagent by neutralizing the HCl present with NaOH, and diluting with water. The exact concentration was determined by a procedure based on Seyewitz & Gibello's method [1904], or by oxidation with H_2O_2 and titration of the acid formed.

***l*-Ascorbic acid, d-glucoscorbic acid and reductic acid**—see Snow & Zilva [1941].

Reductone and dihydroxymaleic acid—see Snow & Zilva [1938].

d-Araboascorbic acid—kindly presented by Prof. E. L. Hirst. Brownish white crystalline powder. Purity 89% by indophenol titration.

6-Desoxy-*l*-ascorbic acid—kindly presented by Prof. T. Reichstein. Pure white crystals. Purity 100% by titration.

6-Trityl-*l*-ascorbic acid—prepared by the method of Vargha [1933]. The product prepared by us was similar in characteristics to that obtained by Vargha but its purity as determined by indophenol titration was only 70%.

5:6-Isopropylene-*l*-ascorbic acid—prepared by Vargha's method [1932]. Crude product recrystallized four times from acetone. Pure white; m.p. 208° (decomp.). Unlike Vargha, we found that this compound was not readily hydrolyzed in water. Vargha based his conclusion on the fact that in aqueous solution the molecular rotation of the isopropylene compound was identical with that of ascorbic acid. We also found these values to be similar but further work showed that this agreement was fortuitous. Thus when aqueous solutions were diluted with four volumes of ethanol the rotations of ascorbic acid and its acetone derivative were markedly different. It is seen (Table 1) that

Table 1. Optical rotation of 5:6-isopropylene-*l*-ascorbic acid in aqueous and in alcoholic solutions

(All measurements were made at 21° with light of wavelength 546 mμ.)

Solvent	Time before addition of alcohol to aqueous solution (hr.)	<i>l</i> -Ascorbic acid		5:6-Isopropylene- <i>l</i> -ascorbic acid	
		Concentration (g./100 ml.)	[α]	Concentration (g./100 ml.)	[α]
Water	—	1.33	25°	1.63	27°
80% ethanol	0	0.27	52°	0.33	13°
	3½	—	—	0.33	14°
	7	—	—	0.33	16°

even after 7 hr. the change in rotation of the latter was so small that it was probably within the limits of the experimental error. The action of HCHO upon this compound could therefore be studied without any serious interference due to hydrolysis.

Buffer solutions.

	Vol. used to prepare buffers of pH	
	1.5	3.5
10% CCl_3 .COOH solution	500 ml.	500 ml.
Citric acid (Analar)	24.7 g.	62.5 g.
10N NaOH (Analar)	22.6 ml.	56.8 ml.
Water to	1 l.	1 l.

The pH of each solution was checked electrometrically after addition of 27.5 ml. water and 5 ml. 6M HCHO solution/25 ml. buffer solution. If necessary, small additions of HCl or NaOH were made to the stock solutions to bring the pH after dilution to exactly 1.50 or 3.50.

Procedure. 25 ml. buffer solution and 30 ml. HCHO solution of the requisite concentration, contained in a 100 ml. flask, were placed in a thermostat and allowed to attain the temperature of the bath. As a precaution against atmospheric oxidation a current of O_2 -free N_2 was passed through the reaction mixture during the experiment. An aqueous solution of ascorbic acid or other reducing compound (2.5 ml.) was added to make the initial concentration in every case 0.46 mM. Samples of the mixture were then withdrawn at intervals, acidified with HCl to arrest further reaction, and titrated with 2,6-dichlorophenolindophenol. The initial titration value of the reducing compound was determined separately. The results given are in every case the mean of at least two experiments and are expressed as percentages of the original concentration of the reactant.

Special modifications of this procedure were found necessary in the case of certain of the reducing substances employed. The estimation of reductone by direct titration is unsatisfactory because the rate of reduction of indophenol is too slow for the end-point to be easily observed. The reductone solutions were therefore added to an excess of acidified indophenol solution, allowed to stand for 1 min. and back-titrated with ascorbic acid solution stabilized with KCN, NaCl and acid. This method, although not entirely satisfactory in the presence of large concentrations of HCHO, was definitely preferable to the direct titration procedure.

Dihydroxymaleic acid in aqueous solution loses its reducing capacity on standing. In order to avoid this it was dissolved in absolute ethanol before addition to the buffer-HCHO mixture. It was also necessary to dissolve 6-trityl ascorbic acid in ethanol, owing to its low solubility in water.

In the case of alloxantin special precautions had to be taken to avoid oxidation by atmospheric O_2 . Water and buffer solutions were boiled and cooled in a stream of N_2 and the reaction was conducted under strictly anaerobic conditions.

RESULTS

The kinetics of the reaction between formaldehyde and l-ascorbic acid

The reaction was studied over a range of temperatures and HCHO concentrations. It has been deduced from the data (Table 2) that the reaction is reversible and proceeds according to the equation $A + F \rightleftharpoons AF$ (A = ascorbic acid, F = HCHO, and AF = the complex formed from equimolecular proportions of the reactants). The forward reaction here is bimolecular and the opposing reaction unimolecular. Owing, however, to the high concentration of HCHO relative to that of the ascorbic acid present (23 molar equivalents at the lowest concentration used), the concentration of the former reactant may be considered constant, and the forward reaction therefore behaves as a reaction of the first order, with

$$\frac{dx}{dt} = k_1 (a - x) [F] - k_2 x.$$

From this may be deduced

$$k_1 [F] + k_2 = \frac{1}{t} \ln \frac{x_e}{x_e - x}$$

(a = initial concentration of ascorbic acid; x = amount combined in time t ; x_e = amount of ascorbic acid combined at equilibrium; k_1 = velocity constant of forward bimolecular reaction; k_2 = velocity constant of opposing unimolecular reaction; and $[F]$ = concentration of HCHO). From the equilibrium equation the equilibrium constant (K) is obtained as follows:

$$K = \frac{[AF]}{[A][F]} = \frac{k_1}{k_2}.$$

From the experimental results, values for K and $k_1 [F] + k_2$ could be derived, and hence the constants k_1 and k_2 could be calculated.

It will be seen from Table 2 that the figures for K and k_1 at a given temperature were constant within the limits of the experimental error, except for a slight deviation at the highest HCHO concentration at 30°; all values of K for the highest HCHO concentrations, however, must be regarded as somewhat unreliable owing to the smallness of the titration values from which they were derived. The general constancy of K and k_1 demonstrates that the reaction follows the postulated course over a considerable range of HCHO concentrations. An independent check was provided by plotting the values of $k_1 [F] + k_2$ against the HCHO concentration in the case of experiments carried out at 30.3°. The points lay along a straight line intersecting the axis at a point representing the value of k_2 . The figure obtained in this way was 0.03, which was in good agreement with the value of 0.028 calculated from the equilibrium constant.

The variation of the velocity constants with temperature agreed with Lugg's results. At a HCHO concentration of 0.526 M, $k_{25^\circ}/k_{15^\circ} = 2.8$ ($k = k_1 [F] + k_2$). Lugg's corresponding figure was 3.0. In the presence of this large excess of HCHO, k_2 was almost negligible and values of

$$\log (k_1 [F] + k_2)$$

plotted against the reciprocal of the absolute temperature were found to lie on a straight line (Fig. 1). The same diagram also shows a similar rectilinear relationship when k_1 , k_2 and K are plotted against $1/T$. The change in the equilibrium constant is of practical significance when the method is used under extreme conditions of temperature. The error introduced by temperature changes is much more serious at low HCHO concentrations, and it is probable that Lugg's failure to detect the variation was due to the high HCHO concentration present in his experiments.

Lugg's method is based on the fact that the velocity of combination of HCHO with ascorbic acid is much lower at pH 1.5 than at pH 3.5. In studying the course of the reaction at pH 1.5, only the early stages were considered by us, because of

Table 2. *The influence of temperature on the rate of combination, at pH 3.5, of ascorbic acid with varying concentrations of formaldehyde*
Initial ascorbic acid concentration, 0.46 mM. Equilibrium and velocity constants are expressed in mole, litre, minute units.

Temp. (°C.)	HCHO conc. (M)	Uncombined ascorbic acid after <i>T</i> min. (%)												Ascorbic acid com- bined at equilibrium (%)	Equi- librium constant <i>K</i>	Mean $k_1[F] + k_2$		k_1	$k_2 \times 10^3$
		1	2	3	4	6	8	10	20	30	40	50	60						
30.3	0.526	1 54.0	2 29.3	3 16.0	4 8.7	6 2.8	8 2.0	10 2.0	20 1.8	30 1.8	40 1.8	50 1.8	60 1.8	98.0	(92)	1.30	(2.43)		
	0.132	1 51.7	2 28.1	3 17.1	4 12.0	6 7.4	8 7.1	10 7.1	20 —	30 —	40 —	50 —	60 —	92.9	100	0.37	2.60		
	0.0526	1 50.8	2 31.8	3 22.8	4 19.7	6 16.9	8 16.8	10 16.8	20 16.8	30 16.8	40 16.8	50 16.8	60 16.8	83.2	94	0.17	2.76		
	0.0263	1 54.5	2 37.7	3 32.1	4 29.6	6 28.6	8 28.4	10 28.6	20 28.6	30 28.6	40 28.6	50 28.6	60 28.6	71.8	97	0.099	2.72		
	0.0105	1 86.4	2 77.2	3 70.0	4 63.9	6 57.4	8 54.0	10 50.0	20 49.1	30 48.0	40 47.9	50 47.9	60 47.9	52.1	104	0.058	2.83		
		Mean																	
25	0.526	1 45.5	2 21.9	3 10.1	4 5.8	6 2.4	8 1.6	10 1.5	20 —	30 —	40 —	50 —	60 —	98.4	(115)	0.80	1.50		
	0.132	1 78.4	2 63.8	3 43.6	4 20.5	6 12.3	8 6.6	10 5.4	20 5.5	30 5.5	40 5.5	50 5.5	60 5.5	94.6	133	0.23	1.64		
	0.0263	1 83.2	2 71.5	3 54.3	4 36.6	6 26.9	8 23.4	10 22.4	20 —	30 —	40 —	50 —	60 —	77.6	132	0.057	1.67		
		Mean																	
	0.526	1 62.8	2 38.4	3 24.0	4 15.7	6 9.9	8 6.9	10 5.1	20 4.3	30 4.3	40 4.3	50 4.3	60 4.3	98.5	(125)	0.49	0.92		
	0.132	1 77.3	2 58.6	3 38.1	4 24.5	6 16.2	8 9.0	10 5.1	20 4.5	30 4.5	40 4.5	50 4.5	60 4.5	95.7	171	0.13	0.96		
15	0.0263	1 80.9	2 67.8	3 50.6	4 36.9	6 24.0	8 19.9	10 18.7	20 —	30 —	40 —	50 —	60 —	81.3	166	0.031	0.96		
		Mean																	
	0.526	1 73.2	2 54.9	3 43.5	4 32.8	6 19.4	8 11.6	10 8.0	20 3.0	30 1.1	40 1.1	50 1.1	60 1.1	99.0	(189)	0.29	0.54		
	0.132	1 76.1	2 58.1	3 34.7	4 24.5	6 16.2	8 9.0	10 5.1	20 3.4	30 3.4	40 3.4	50 3.4	60 3.4	96.6	218	0.071	0.52		
	0.0263	1 70.5	2 49.9	3 37.0	4 28.6	6 20.3	8 16.3	10 15.2	20 —	30 —	40 —	50 —	60 —	85.3*	220	0.015	0.48		
		Mean																	
10	0.526	1 81.0	2 72.0	3 59.8	4 51.7	6 27.7	8 15.4	10 8.0	20 0.8	30 0.8	40 0.8	50 0.8	60 0.8	99.2	(247)	0.17	0.31		
	0.132	1 83.8	2 70.9	3 50.5	4 29.6	6 14.2	8 6.4	10 3.6	20 2.9	30 2.9	40 2.9	50 2.9	60 2.9	97.1	251	0.044	0.32		
	0.0263	1 76.7	2 60.6	3 32.0	4 20.1	6 15.7	8 13.7	10 —	20 —	30 —	40 —	50 —	60 —	87.8*	273	0.010	0.83		
		Mean																	

* Extrapolated.

(Figures in brackets not included in mean.)

the considerable time required to attain equilibrium. It followed that, at the high concentration of HCHO employed in all the experiments, the quantity of

Fig. 1. Reaction between ascorbic acid and formaldehyde. Variation of equilibrium and velocity constants with temperature. (The data were derived from those given in Tables 2 and 3.) T = absolute temp. Curve A: pH, 1.5. $4 + \log(k_1[F] + k_2)$. Curve B: pH, 3.5. $2 + \log(k_1[F] + k_2)$. Curve C: pH, 3.5. $2 + \log k_1$. Curve D: pH, 3.5. $4 + \log k_2$. Curve E: pH, 3.5. $\log K$ (equilibrium constant).

ascorbic acid combined at equilibrium could be assumed to be 100%, without the introduction of a serious error. The calculation was further simplified by neglecting k_2 , which is very small under these conditions, and by accepting the expression $k_1[F] + k_2$ as the velocity constant (k) of a first

order reaction. The validity of these simplifications was demonstrated in the case of the experiments at 30°, where measurements were continued almost to the point of equilibrium. It will be seen (Table 3) that the amount of ascorbic acid remaining was even smaller than under similar conditions at pH 3.5. When $\log k$ was plotted against $1/T$ (Fig. 1), the graph was a straight line with a slope only slightly different from that obtained for the same HCHO concentration at pH 3.5; $k_{25}/k_{15} = 3.1$.

It may be noted that we found a much greater difference between the velocities of reaction at pH 3.5 and 1.5 than that reported by Lugg. He found the velocity constants at pH 1.5 to be 0.03 times those at pH 3.5, whilst the ratio in our experiments was approximately 0.012 (varying slightly with temperature). From the figures given in his paper it would appear that the cause of the discrepancy lies in differences between the velocity constants obtained for the reaction at pH 1.5. The disagreement may possibly be explained by the difference in the composition of the solutions; in our experiments trichloroacetic acid was present, whilst Lugg employed metaphosphoric acid.

The kinetics of the reactions of formaldehyde with substances chemically related to l-ascorbic acid

The substances investigated were: *d*-araboascorbic acid, which differs from *l*-ascorbic acid only in the configuration of carbon 4; *d*-glucoascorbic acid, a seven carbon analogue; reductic acid, an alicyclic compound containing a ketodienol group; reductone and dihydroxymaleic acid, both acyclic compounds with a dienol group, and alloxantin.

d-Araboascorbic and *d*-glucoascorbic acids. The velocity of reaction of these two compounds with HCHO (Tables 4 and 5) was similar to that of *l*-ascorbic acid, both at pH 1.5 and 3.5 (cf. Tables 2 and 3). At the higher pH value the reaction rapidly reached an equilibrium, but the

Table 3. The influence of temperature on the rate of combination of ascorbic acid with formaldehyde at pH 1.5

Formaldehyde concentration, 0.52M. Initial ascorbic acid concentration, 0.46mM.

Temp. °C.	Uncombined ascorbic acid after T min. (%)									
	Velocity constant $k = (k_1[F] + k_2)10^3$									
29.7	T	4	8	12	16	20	60	120	180	270
	%	92.9	87.2	82.1	76.4	71.0	35.1	13.3	5.5	2.1
	k	(18.4)	17.3	16.7	17.0	17.4	17.8	(19.4)	17.3	17.1
25	T	5	10	15	20	25	30	35		
	%	94.1	89.0	84.1	79.5	75.4	71.3	67.5		
	k	(12.0)	11.7	11.5	11.5	11.3	11.3	11.3		
20	T	10	20	30	45	50	60	70	80	
	%	93.8	88.3	83.4	76.5	74.5	69.3	65.3	62.4	
	k	(6.45)	6.22	6.07	5.94	5.90	6.11	6.09	5.91	
15	T	20	40	60	80	100	120			
	%	92.9	86.6	80.5	75.4	70.8	66.2			
	k	3.68	3.57	3.61	3.54	3.46	3.44			
10	T	30	60	90	120	150	180			
	%	94.6	88.7	85.0	79.8	75.1	70.7			
	k	1.84	2.00	1.82	1.88	1.90	1.93			
										Mean

* % ascorbic acid at equilibrium = 1.1 (extrapolated).

Figures in brackets not included in mean.

Table 4. *Reaction of various reducing substances with formaldehyde at pH 3.5*

Initial concentration of reducing compounds, 0.46M. Temp. 25°

Reducing compound	HCHO conc. = $[F]$ (M)	Uncombined reducing compound after T min. (%)											Reducing compound combined at equilibrium = x_e (%)	$x_e [F]^{-1}$
		Velocity constant $k = k_1 [F] + k_2$												
		T	$\frac{1}{2}$	1	2	3	4	8	12	20				
<i>d</i> -Glukoascorbic acid	0.526	% k	58.9 (1.07)	38.5 0.96	15.6 0.94	6.0 0.97	2.9 0.94	0.7 Mean	0.5 0.95	0.5	—	—	99.5	
<i>d</i> -Araboascorbic acid	0.526	% k	64.2 (0.92)	45.8 (0.81)	24.5 0.74	13.2 0.73	7.8 0.72	3.1 Mean	2.4 0.73	2.4	—	—	97.6	
Reductic acid		T	$\frac{1}{2}$	1	2	4	8	15	30	60	90	120		
	4.8	%	75.4	73.7	73.7	71.7	68.4	65.9	59.9	51.9	47.1	42.9	27	12
	2.4	%	83.6	83.6	84.1	81.1	79.8	78.6	75.1	70.1	67.9	65.9	18.5	12
	1.2	%	89.5	88.0	88.0	87.2	86.7	85.7	84.4	82.9	79.9	78.7	12.5	11
	0.60	%	94.4	93.2	93.2	93.4	92.7	90.9	91.4	90.2	88.9	87.9	8	10
Reductone		T	1	2	4	8	12	16	23					
	4.84	%	77	69	47	26	21	15	11					
		T	10	15	20	25	30	40	60	100	150			
	0.605	%	74	60	—	48	42	34	20	11	7			
	0.151	%	93	88	86	84	79	73	62	46	36			
Dihydroxymaleic acid		T	1	2	4	6	8	12	16	20	30	40		
	4.84	%	91.6	77.5	62.5	48.6	38.5	24.8	15.2	9.9	2.5	0		
		T	2	4	8	12	20	30	40	60	80	100		
	0.605	%	93.7	88.7	80.8	72.2	55.2	41.5	30.9	17.5	10.1	6.0		
		T	6	15	23	30	40	50	60	70	90	110		
	0.151	%	88.8	72.5	63.9	53.2	43.5	35.1	28.5	24.4	15.8	10.9		
		T	15	30	45	60	80	100						
	Q	%	78.8	62.2	49.5	37.6	29.0	22.7						
5:6-Isopropylene- <i>l</i> -ascorbic acid		T	$\frac{1}{2}$	1	2	4	8	15	30	60	90	120		
	0.52	%	78.2	67.4	57.4	51.5	49.5	47.8	44.5	40.1	39.1	37.5	48.5	67
	0.26	%	86.5	80.3	71.1	65.6	62.3	60.4	57.8	54.0	51.0	48.8	36.5	72
	0.13	%	92.0	88.7	83.2	79.1	76.2	74.0	71.2	68.3	64.7	62.3	25	69
	0.065	%	94.0	92.4	89.3	86.2	84.6	82.5	78.7	76.0	74.4	71.6	19	75

(Figures in brackets not included in mean.)

Table 5. *Reaction of various reducing substances with formaldehyde at pH 1.5*

Initial concentration of reducing substances, 0.46 mM.

Reducing compound	HCHO conc. (M)	Temp. (°C.)	Uncombined reducing compound after T min. (%)							Mean
			Velocity constant $k = (k_1 [F] + k_2) 10^3$							
			T	5	10	15	20	25	30	
d -Glukoascorbic acid	0.52	25	%	94.2	89.0	84.7	79.2	74.9	69.6	11.7
			k	12.0	11.7	11.1	11.6	11.5	12.1	
Reductic acid	0.52	30	%	93.9	90.5	91.2	89.8	88.8	86.1	
Reductone	0.52	30	%	98	93	90	85	74	70	
Dihydroxymaleic acid			T	10	20	30	50	70	90	
	0.52	30	%	73.4	51.5	38.2	21.1	11.4	8.9	
	0		%	79.0	55.7	44.8	24.6	18.6	14.0	

amounts of the reducing substances combined differed somewhat from the corresponding values for *l*-ascorbic acid. The significance of this difference was, however, uncertain in view of the fact that neither of the two compounds was completely pure.

Reductic acid. The reaction of this compound at pH 3.5 was studied over a range of HCHO concentrations. As will be seen from Table 4, there was a rapid initial reaction which was followed by a very much slower one. The two stages can be clearly seen if the titration values are plotted against time. The rapid reaction is then represented by

the steep initial part of the curve, the slope of which decreases sharply as this reaction ceases, and the slow reaction is indicated by the lower gradient of the curve as time proceeds. By extrapolating the flat portion of the curve back to the axis it was possible to determine the 'equilibrium' value of the initial reaction. The amount of reductic acid combined at this primary 'equilibrium' was far less than the equilibrium value for ascorbic acid at a corresponding HCHO concentration. The amount of reductic acid combined at 'equilibrium' varied with the HCHO concentration in a manner quite different from that found

with ascorbic acid. Owing to the uncertainty of the nature of the reaction involved it was not possible to calculate an equilibrium constant. An empirical relationship was, however, found to exist between the HCHO concentration and the percentage of reductic acid combined at equilibrium, i.e. $x_e \propto \sqrt{[F]}$ (symbols as in preceding section), as shown by the approximate constancy of the product $x_e [F]^{-\frac{1}{2}}$ (Table 4). The significance of this result is not apparent, and the question of the mode of combination of reductic acid with HCHO remains unsettled. It is, however, evident that at least two reactions are involved, one of which proceeds at a speed comparable with that of the ascorbic acid-HCHO reaction and quickly reaches an equilibrium.

It is probable from the figures given in Table 5 that the course of the reaction at pH 1.5 is quite similar to that at pH 3.5, though the rate of combination is very much slower.

Reductone. Lugg included this substance in the class of compounds which do not condense appreciably with HCHO either at pH 3.5 or 1.5. The results of our experiments at pH 3.5, with different HCHO concentrations, showed, however, that although the velocity of combination of reductone with HCHO was much lower than in the case of ascorbic acid, it was nevertheless significant. This was also found by Mapson [1943]. As the figures for this compound lacked the precision attained with other reducing compounds, the conclusions about the kinetics of this reaction were not so well defined. It was apparent, however (Table 4), that the loss of reductone was approximately exponential and continued until all, or at least a very large part, of this reactant was exhausted. It may be noted that the initial velocity of the reaction was approximately proportional to the concentration of HCHO present. The behaviour of reductone was thus completely different from that of reductic acid. Reductone also reacted with HCHO at pH 1.5, although under these conditions the rate of combination was very slow indeed (Table 5).

Dihydroxymaleic acid. Experiments on this compound were complicated by the instability of aqueous solutions of the substance, both at pH 3.5 and 1.5, even under completely anaerobic conditions, and a comparatively rapid loss of reducing power occurred without the addition of HCHO. The data (Table 4) show that at pH 3.5 the rate of loss of dihydroxymaleic acid in the presence of 0.15M HCHO was only slightly more rapid than in the absence of the latter. Higher concentrations of HCHO, however, caused the reaction to proceed more swiftly, and at a concentration of 4.84M no reducing capacity remained after 40 min. It thus seems possible that, apart from its spontaneous decomposition, the behaviour of dihydroxymaleic acid towards HCHO resembles that of reductone. From Table 6 it will be seen that at pH 1.5 the loss of the compound due to spontaneous decomposition occurred at a rate similar to that observed at pH 3.5, and was only slightly accelerated by HCHO at a concentration of 0.53M.

Alloxantin. Alloxantin combined very rapidly with HCHO both at pH 3.5 and 1.5; at a concentration of 0.52M HCHO, with pH 1.5, no reduction could be detected after 30 sec. at 25°. This compound thus undoubtedly belongs to Class 1 of Lugg's classification, which includes substances that readily condense with HCHO at pH 1.5.

Corrections for certain interfering substances

It is seen from the above that Lugg's method is incapable of distinguishing between *l*-ascorbic acid and its close analogues, and consequently no corrections could be made

for their presence. On the other hand, *l*-ascorbic acid can be determined with some degree of accuracy in the presence of high concentrations of reductic acid, reductone or dihydroxymaleic acid if the necessary corrections are made.

In cases where the value obtained after treatment with HCHO at pH 3.5 is small in relation to that obtained by direct titration, no further correction is needed. If, on the other hand, this value is large, corrections for substances behaving like reductone and reductic acid are called for.

If the presence of reductone be suspected, it can be ascertained by oxidizing the extract with indophenol and subsequently reducing it with H_2S . By this treatment only a small fraction of the original reducing value of the reductone is regenerated, whilst the dehydroascorbic acid is completely reduced to ascorbic acid. However, this procedure may not diminish the error due to other substances which behave like reductone in the HCHO technique, and a general correction formula can be introduced in such cases.

The velocity of combination of reductone with HCHO is much lower than that of ascorbic acid at pH 3.5, but in the time required to bring the ascorbic acid reaction to within 0.5% of equilibrium approximately 17% of the reductone would combine. If, however, the experiment is repeated by doubling the reaction time, approximately twice as much reductone will combine, since the course of the reaction is almost linear in the early stages (Fig. 2);

Fig. 2. Reaction of various reducing substances with formaldehyde at pH 3.5. Initial concentration of reducing substances, 0.46mM. HCHO concentration, 0.52M. Temp. 25°.

in both cases the ascorbic acid-HCHO reaction may be considered to have reached equilibrium. Thus the following method of calculation may be employed if reductone or substances behaving like it are present.

$$v_1 = v + w,$$

$$v_2 = v \left(1 - \frac{x_e}{100} \right) + w(1 - p),$$

$$v_3 = v \left(1 - \frac{x_e}{100} \right) + w(1 - 2p),$$

whence
$$v = (v_1 - 2v_2 + v_3) \frac{100}{x_e}$$

(v_1 = corrected titration after reaction with HCHO at pH 1.5; v_2 = titration after reaction at pH 3.5 for standard

time (t); v_2 =titration after reaction at pH 3.5 for time $2t$; v =titration equivalent of actual ascorbic acid present; w =titration equivalent of reductone present; x_e =percentage of ascorbic acid combined at equilibrium, and p =fraction of total reductone combined in time t).

This procedure is similar to that adopted by Mapson [1943]. Mapson's modification has the advantage that it does not require special care in the adjustment of the pH and the concentration of HCHO, and is independent of any changes in the reaction velocity at pH 3.5. On the other hand, it requires more material and time for the estimation, and above all, as in the case of Lugg's original method, no correction is made for the error that might arise from the presence of reductic acid.

As the initial velocity of combination of reductic acid with HCHO is comparable with that of ascorbic acid, the procedure recommended for use with reductone and like substances is of no value in this case. However, use may be made of the fact that the amount of reductic acid combined at 'primary equilibrium' is proportional to $\sqrt{[\text{HCHO}]}$. After the usual HCHO technique at pH 3.5 is carried out, the procedure is repeated with one-fourth of the concentration of HCHO, the reaction time being suitably increased so as to allow the ascorbic acid-HCHO system to reach equilibrium. In this second titration the percentage of reductic acid combined will be approximately one-half of that in the first titration.

$$v_1 = v + y,$$

$$v_2 = v \left(1 - \frac{x_e}{100} \right) + y(1 - q),$$

$$v_4 = v \left(1 - \frac{x_e'}{100} \right) + y(1 - \frac{1}{2}q),$$

whence
$$v = \frac{100(v_1 - 2v_4 + v_2)}{2x_e' - x_e}$$

(v_2 =titration after reaction at pH 3.5 with standard HCHO concentration (c); v_4 =titration after reaction at pH 3.5 with HCHO concentration 0.25 c ; y =titration equivalent of reductic acid present; q =fraction of reductic acid combined at HCHO concentration c ; x_e =percentage of ascorbic

acid combined at equilibrium with HCHO concentration c ; x_e' =percentage of ascorbic acid combined at equilibrium with HCHO concentration 0.25 c ; v_1 and v have the same significance as before).

Values for the reaction times and the percentage of ascorbic acid combined at equilibrium for the two HCHO concentrations are given in Table 6, in order that this correction may be applied.

The presence of a substance like dihydroxymaleic acid, which decomposes spontaneously both at pH 3.5 and 1.5, would undoubtedly lead to erroneous results in applying Lugg's method, if the extracts were not previously treated with H_2S . If, however, the solutions are allowed to stand in the presence of H_2S , this interfering substance will decompose before the estimation is commenced, and error due to its presence will thus be avoided.

The structure of the formaldehyde-ascorbic acid complex

It has already been seen from the kinetics of the reaction, that HCHO and ascorbic acid combine in equimolar proportions. It is therefore probable that the HCHO molecule has two points of attachment in the ascorbic acid molecule. An attempt to isolate the complex, by evaporating concentrated aqueous solutions or by fusing ascorbic acid with polymers of HCHO, did not meet with much success. Rigorous drying gave almost colourless glassy solids which deliquesced immediately on exposure to air and which contained a small amount of unchanged ascorbic acid. When their aqueous solutions were made alkaline and immediately acidified, a large part of the indophenol titration value of the combined ascorbic acid was regenerated. This will be seen later to be characteristic of the complex. Thus these impure solid products contained a considerable proportion of material which behaved in a manner similar to the ascorbic acid-HCHO complex produced in solution. Purification of the solids was rendered difficult by their extremely deliquescent nature, and, in view of the probable complexity of the ascorbic acid-HCHO reaction, further attempts to isolate the complex were felt to be unprofitable. The problem of structure had therefore to be attacked by indirect methods.

The fact that after combination with HCHO the ascorbic acid loses its reducing properties suggests that either or both of the OH groups attached to C_2 and C_3 are involved. Our first object was therefore to inquire into the nature of this linkage. If the condensation were to involve C_3 , it should lead to a decrease in the acidic properties of ascorbic acid, since the H atom in this position is probably the one which is mainly ionized [Reichstein, Grüssner & Oppenauer, 1934; Haworth & Hirst, 1934; Haworth, Hirst & Smith, 1934]. In such a case the pH of an unbuffered mixture of HCHO and ascorbic acid should increase as the reaction proceeds. To test the point the following experiment

Table 6. *Time required for the combination of ascorbic acid with formaldehyde at pH 1.5 and 3.5*

[F]=concentration of HCHO in reaction mixture.

x_e =percentage of ascorbic acid combined at equilibrium.

At pH 1.5, times given are those required to produce the percentage loss indicated.

At pH 3.5, times given are those required to reach 99.5% equilibrium value.

Temp. (° C.)	pH 1.5. Time with [F]=0.52M		pH 3.5			
	1% loss (sec.)	5% loss (min.)	[F]=0.52M		[F]=0.13M	
			Time (min.)	x_e	Time (min.)	x_e
30	35	3	4	98.2	15	93.0
25	60	5	7	98.5	24	94.4
20	100	8.5	11	98.9	40	95.6
15	170	15	19	99.1	68	96.5
10	310	26	32	99.3	119	97.3

(The data are derived from those in Tables 2 and 3.)

was carried out. 20 ml. 6M neutral HCHO solution were added to a solution of 0.5 g. ascorbic acid in 180 ml. water at room temperature. The pH of the solution was determined initially and after various intervals by means of a glass electrode system. Simultaneously samples of the mixture were titrated with indophenol. In correlating the pH value and the ascorbic acid concentration of the reacting solution, use was made of a curve previously constructed by measuring the pH of aqueous solutions of known strengths of ascorbic acid. It will be seen from Fig. 3 that although the observed pH values were

Fig. 3. Changes in pH during reaction between ascorbic acid and formaldehyde. Reaction mixture—0.5 g. ascorbic acid, 180 ml. water, 20 ml. 6M neutral HCHO solution. Temp. 20°. Curve A: log ascorbic acid concentration (mg./100 ml.), from indophenol titrations. Curve B: pH calculated from ascorbic acid concentration. Curve C: pH measured electrometrically. Curve D: titration of 5 ml. portions with N/20 NaOH.

somewhat higher than those calculated from the titration values, the changes occurring as the reaction proceeded followed a very similar course in each case. The agreement was quite satisfactory in view of the ease with which the pH of an unbuffered solution may be affected by extraneous substances. The higher pH values were in all probability due to the presence of HCHO. In our opinion this experiment produces sufficient evidence that C_3 is one point of linkage.

In order to determine the position of the second point of attachment, the behaviour of certain derivatives of ascorbic acid containing substituents in positions 5 and 6 was investigated. The compounds employed were 6-trityl-, 6-desoxy- and 5:6-isopropylene-*l*-ascorbic acid, and the reaction of each of these substances with HCHO at pH 3.5 was followed up by titration as before. It will be seen (Fig. 2) that the behaviour of the three compounds was remarkably similar, and was in sharp contrast

to that of ascorbic acid at the same concentration of HCHO. The course of the reaction was not unlike that observed with reductic acid, though the amount of reactant combined during the initial rapid reaction was much greater at a given HCHO concentration in the case of the 6-substituted compounds. The reaction with 5:6-isopropylene-ascorbic acid was investigated over a range of concentrations of HCHO. As in the case of reductic acid the expression $x_e[F]^{-1}$ was found to be approximately constant (Table 4). Since the behaviour of the three compounds was very similar in spite of the different nature of the substituents, it may be inferred that the contrast between the course of their reactions and that with ascorbic acid was probably not due to any change in the reactivity of the dienol H atoms, but to the blocking of position 6. Also since the 5:6 substituted isopropylene derivative behaved exactly like the 6-substituted compounds, linkage at position 5 may be excluded. It thus seems probable that the formation of the HCHO complex involves the 3 and the 6 positions of the ascorbic acid molecule. The possibility that the presence of HCHO might stabilize an abnormal tautomeric form of ascorbic acid having an oxygen bridge attached at C_6 (cf. the structure of isodimethyl ascorbic acid [Micheel & Schulte, 1935; Micheel & Bischoff, 1936; Haworth, Hirst, Smith & Wilson, 1937]) appears to us very unlikely.

In all these experiments it must be emphasized that we have been concerned only with the HCHO condensations which affect the reducing power of ascorbic acid. A few experiments were carried out in an attempt to follow the course of the reaction between ascorbic acid and HCHO by observation of the changes in the optical rotation. The results indicated that the whole reaction system was more complicated than that indicated by titration methods. It is quite probable that other reactions may occur, leading to products which are still capable of reducing indophenol. As the presence of such compounds would have no bearing on the method, the matter was not investigated further.

In connexion with the properties of the ascorbic acid-HCHO complex, we should like to draw attention to a point of interest. In the investigation of the change in pH of the unbuffered ascorbic acid-HCHO solution in the above experiment, samples of the solution were titrated with NaOH (phenolphthalein indicator). Apart from a slight initial rise the titration values remained constant (Fig. 3) throughout the experiment. It would appear from this that the ascorbic acid-HCHO complex is very rapidly decomposed by alkali. This idea was confirmed by the fact that when the alkali-titrated solution was acidified and titrated with indophenol the quantitative regeneration of ascorbic acid was established.

The influence of various substances on the velocity of combination of ascorbic acid and formaldehyde

Since Lugg's method depends upon the rate of combination of ascorbic acid with HCHO at pH 3.5 and 1.5, it is evident that the accuracy of the method would be diminished by the presence of substances capable of influencing the velocity of the reaction. At pH 3.5 the reaction is allowed to reach equilibrium. The times suggested by Lugg are probably greater than is necessary to attain this condition and consequently the possibility of error is reduced. Thus a moderate decrease in the reaction

Fig. 4. The influence of various substances on the rate of combination of ascorbic acid with formaldehyde at pH 1.5. Initial concentration of ascorbic acid, 0.46 mM. Temp. 25°. HCHO added at zero time. Curve A: • No addition; ⊙ 10 mM. creatine added; ⊖ 10 mM. tyrosine added; ⊕ 10 mM. asparagine added; ⊗ 10 mM. leucine added. Curve B: ▽ 10 mM. glycine added. Curve C: ◇ 20 mM. glycine added. Curve D: × 40 mM. glycine added. Curve E: △ Loss of added ascorbic acid from extract of canned meat and vegetables adjusted to pH 1.5.

velocity would not be serious, whilst an increase would have no effect upon the result. At pH 1.5, on the other hand, the reaction is allowed to proceed until a definite fraction of the ascorbic acid present (5% in Lugg's procedure) has combined with HCHO, and consequently any variation in the velocity of reaction at this pH would cause some error in the results. This error might be considerable if the reaction were accelerated. We found that when ascorbic acid was added to extracts of certain canned products the rate of reaction with HCHO at pH 1.5 was in fact considerably higher than in pure buffer solutions. Evidently this acceleration was produced by the presence of interfering substances in the extract and we therefore considered it advisable to investigate the influence of some

compounds likely to be present in such extracts, as well as that of compounds capable of reacting with HCHO. For this purpose the substances were dissolved in buffer solution containing ascorbic acid and the HCHO solution added when the temperature was stabilized. Care was taken to ensure that the pH of the final mixture was 1.50. Results of these preliminary experiments are given in Fig. 4. It will be seen that, at a concentration of 10 mM., any changes in the rate of reaction caused by creatine, *l*-tyrosine, asparagine or leucine were too small to be regarded as significant. On the other hand the presence of glycine (Analar reagent, twice, recrystallized from water), at the same concentration, more than doubled the initial velocity; at higher concentrations the increase was even greater. It must, however, be noted that, whilst the effect with glycine was most marked in the initial stages of the reaction, the increase of velocity observed with the extracts of canned material continued throughout the reaction. The problem requires further investigation, but these results definitely suggest that caution must be exercised in interpreting the results obtained at pH 1.5. If the difference between the direct titration and the values obtained after treatment with HCHO at pH 1.5 is small, no serious error can arise. If, however, the difference is considerable, an inquiry must be instituted in order to ascertain whether this difference is due to the presence of a substance in Class 1 of Lugg's classification or to an increase in the velocity of combination of ascorbic acid with HCHO, brought about by some substance in the extract. It may be noted that many of the substances which are included in Class 1 react almost instantaneously with HCHO at pH 1.5 and therefore the reaction times suggested by Lugg are probably longer than is necessary. We usually allow time for 1% of ascorbic acid to combine instead of 5%. In this way any errors due to changes in the reaction velocity are greatly reduced.

The differential behaviour of the oxidation products of ascorbic acid, reductic acid and reductone

It was mentioned in the introduction that at the time of the appearance of Lugg's paper, we were engaged in elaborating methods for the determination of ascorbic acid in the presence of reductic acid and reductone. Our experiments were based on our observation that the primary oxidation products of the three compounds possessed certain characteristics which were distinctive enough to enable a separation to be effected. Dehydroreductone could be readily distinguished from dehydroascorbic acid by the fact that only a slight indophenol titration was obtained from the former on treatment with

H₂S at pH 3.5–4.5. Ascorbic acid could therefore be determined, in a mixture of these two compounds, by oxidation and reduction. Reductic acid could be distinguished from ascorbic acid by the greater stability of solutions of dehydroreductic acid at a low pH as compared with those of dehydroascorbic acid, and also because in the oxidized state the former reacted with 2,4-dinitrophenylhydrazine more rapidly than the latter. The following is a short description of the procedure adopted.

(a) Sufficient HCl was added to the oxidized solutions to bring the concentration to N/10. The acidified solution was then heated for 1 hr. at 100°, cooled, brought to pH 4.0 by addition of sodium acetate solution, reduced with H₂S and titrated with indophenol, in the usual way, after removal of the excess of H₂S. Under these conditions approximately 89% of the dehydroascorbic acid and only 3% of the dehydroreductic acid were destroyed. In practice the accuracy of the method was limited by the tendency for end-points to be indistinct after the acid treatment. Ascorbic acid mixed with 0.1–10M equivalents of reductic acid could be determined with an error not greater than 10%. For use with tissue extracts it would be necessary to introduce a correction for reductic acid produced, from pectins or pentoses, during the acid treatment, which could be effected by titrating a portion of the solution before reduction with H₂S. It is possible that a more satisfactory procedure could be devised at a lower temperature (40–50°) and higher acid concentration [cf. Penney & Zilva, 1943].

(b) The oxidized solution which contained approximately 0.5mM. dehydroascorbic acid or dehydroreductic acid was buffered at pH 1.5. 10 ml. were treated with 2 ml. 0.2% 2,4-dinitrophenylhydrazine in N HCl, and 1 ml. 2M sodium acetate (to neutralize the acid in the former reagent). The mixture was incubated for 80 min. at 38°, 2 ml. 0.28% aqueous furfural solution added (to remove excess of the hydrazine reagent), and the incubation continued for a further 10 min. Finally 3 ml. acetate buffer were added to bring the pH to 4.0 and the precipitate removed by filtration. The filtrate was reduced with H₂S and subsequently titrated in the usual way. In the hydrazine treatment approximately 6.5% of the dehydroascorbic acid and 96% of the dehydroreductic acid were lost. Ascorbic acid mixed with 0.1–10M equivalents of reductic acid could be determined with an error of less than 4%. In attempting to apply this procedure to extracts of biological materials we encountered difficulty owing to the formation of colloidal solutions of the dinitrophenylhydrazones.

DISCUSSION

There is little doubt that Lugg's procedure greatly improves the specificity of the indophenol titration

for *l*-ascorbic acid. The specificity is, however, not absolute and critical judgement has to be employed in interpreting the results. Apart from the fact that *l*-ascorbic acid cannot be distinguished from its close analogues by this method, every determination must be considered in relation to the circumstances of any given case, in order to correct for the possible presence of interfering substances. These compounds may either belong to a category of compounds capable of reducing indophenol, or may exert an influence on the rate of combination of *l*-ascorbic acid with HCHO.

In applying Lugg's method the following points have therefore to be borne in mind: Lugg assumes that, when equilibrium is reached, 2% of the ascorbic acid remains uncombined. Our results show that the amount of ascorbic acid at equilibrium depends on temperature (Table 6). This point is of practical importance if the determination is carried out at extreme temperatures. Furthermore, the reaction is very sensitive to slight changes in pH. Although fair accuracy can be obtained under favourable conditions with indicators (methyl orange, thymol blue), the use of a glass electrode system for the control of the pH is desirable. It must also be pointed out that a change in pH may occur on the addition of HCHO. The concentration of HCHO is also important, and the strength of the reagent should be determined before use. It is important, as suggested by Lugg, to pass N₂ through the solutions during the determinations.

Under the conditions employed by us, the times required for the reactions at pH 1.5 and at 3.5 differed somewhat from those given by Lugg. In Table 6 are given the times required for 1 or 5% of ascorbic acid to combine with HCHO at pH 1.5. At pH 3.5, the times required for the reaction to proceed to 99.5% of the equilibrium value are given. In practice these times are multiplied by a factor of 1.5 in order to avoid an error due to any slight decrease in the velocity of the reaction that may occur.

Lugg's procedure depends for its accuracy upon the constancy of the velocity of the reaction between ascorbic acid and HCHO. A diminution in the velocity would not be serious unless it were sufficient to prevent the establishment of an equilibrium at pH 3.5 in the time chosen. A considerable acceleration of the reaction, however, would affect the results obtained at pH 1.5. This is a point of practical significance, since it has been found that certain extracts can increase the reaction velocity, and an increase can also be brought about by the addition of glycine. Such errors may be reduced, although not eliminated, by reducing the reaction times employed.

Lugg's method greatly reduces the error in the titration method due to the presence of reductone

or reductic acid, when present in quantities which are small in relation to that of the ascorbic acid present. When larger quantities of these compounds are present, additional corrections must be introduced.

SUMMARY

1. The kinetics of the reaction between HCHO and *l*-ascorbic acid or chemically related substances have been studied.

2. Indirect evidence is produced for the possible structure of the HCHO-*l*-ascorbic acid complex.

3. Lugg's method for determination of *l*-ascorbic

acid has been examined from the point of view of the above experiments, and necessary precautions are stressed.

4. Suggestions for other methods for the determination of *l*-ascorbic acid in the presence of reductic acid and reductone are advanced.

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Studies in Immunochemistry

7. THE ISOLATION FROM HOG GASTRIC MUCIN OF THE POLYSACCHARIDE-AMINO ACID COMPLEX POSSESSING BLOOD GROUP A SPECIFICITY

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Investigations on the nature of the blood group antigens present in human tissues and secretions are greatly restricted owing to the small amounts of active factors present and to the difficulty of obtaining the tissues and secretions in large quantities. An observation of Schiff [1930], however, revealed that commercial peptone contained a considerable quantity of a blood group A factor, and subsequently Schiff & Weiler [1931], Brahn, Schiff & Weinmann [1932] and Schiff [1933] showed that commercial pepsin likewise could serve as a readily available source of this blood group substance. A rich supply of the A-factor was discovered later in hog gastric mucin by Landsteiner & Chase [1936] and by Meyer, Smyth & Palmer [1937]. As a result of their work on the glyco-proteins occurring in gastric mucosa, Meyer *et al.* [1937] isolated from this tissue a polysaccharide that possessed intense A activity and showed that it contained N-acetylglucosamine, and galactose in equimolecular quan-

ties. On the basis of the glucosamine content the product was considered to be only 75 % pure, and it was observed that the material gave a positive Ehrlich diazo-reaction. Landsteiner & Harte [1940] confirmed these observations and extended the investigation to include a closer examination of the non-hexosamine nitrogenous constituent. They concluded that the A-substance consisted largely of carbohydrate but contained also a component rich in amino-acids.

The A-substance in hog gastric mucin is closely related serologically to the human blood group A factor. In view of the ease with which considerable amounts of hog gastric mucin could be obtained, it seemed a particularly suitable material for experimental study, the results of which could be employed subsequently as a basis for the elaboration of a method for the isolation of the blood group substances from human erythrocytes, tissues and tissue fluids. Cellular and tissue antigens, however,