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Spectrophotometric Assay of Ascorbic Acid in Pure Solutions, Blood Plasma and Urine with *peri*Naphthindanetrione Hydrate

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Chemical methods of determining ascorbic acid have replaced almost entirely the earlier biological methods. Most chemical methods are based on the reducing capacity of ascorbic acid. Titration with iodine has proved to be inadequate for the determination of ascorbic acid in natural products, since they contain other reducing substances and since the colour of such products interferes with the determination of the end point of titration. Oxidation of ascorbic acid with the dye 2:6-dichlorophenolindophenol originally suggested by Tillmans (1927), has found extensive use, but in some materials the presence of reducing substances, such as 'reductones' and 'reductic acid' interferes. Several procedures have been devised to minimize these interferences. The specificity of such methods for determination of ascorbic acid is, however, not well established (Snow & Zilva, 1944). Visual titration methods by this dye are not applicable to the study of products containing water or acid-soluble pigments or reducing agents other than ascorbic acid which react with the dye at a rate sufficiently rapid to influence the end point of titration. Photometric methods (Bessey, 1938; Nochberg, Melnick & Oser, 1943) based on the 2:6-dichlorophenolindophenol reagent avoid certain of the limitations of the visual titration techniques. The fact that this dye is readily soluble in xylene makes it possible to use a xylene extraction technique (Pepkowitz, 1943) to eliminate interference from acid-soluble pigments and turbid solutions.

Reaction of derivatives of ascorbic acid with 2:4-dinitrophenylhydrazine permits the determination of ascorbic acid by methods not based upon oxidation-reduction properties. A technique described by Roe & Oesterling (1944) for total and dehydro-ascorbic acid, appears to avoid interference from certain types of reducing materials. Although osazones result from the reaction of sugars with

2:4-dinitrophenylhydrazine, their properties and rates of formation are sufficiently different from those of ascorbic acid derivatives to prevent their interference with the estimation. This method does not prevent interference from 'reductones' and 'reductic acid', but a means of correcting the results due to these substances has been reported (Penney & Zilva, 1945).

In the present work the authors propose a new spectrophotometric method for the estimation of ascorbic acid in pure solutions, blood plasma and urine. This method is based on the colour reaction obtained by the action of *perinaphthindanetrione* hydrate (IIa) and its derivative 2-nitro*perinaphthindanetrione* hydrate (IIb) with ascorbic acid. Preliminary spectroscopic examination of these two reagents has been carried out. Further study in this field is in progress, and details will be published later.

Nature of the colour reaction. Moubasher (1948) and Moubasher & Sina (1950) found that when solutions of *perinaphthindanetrione* hydrate (IIa) (cf. Errera, 1913, 1914) or 2-nitro*perinaphthindanetrione* hydrate (IIb) (cf. Calderaro, 1916) and ascorbic acid (I), are mixed at room temperature, a deep red colour rapidly develops due to the formation of dihydroxy*perinaphthindenone* (IVa) or dihydroxy-2-nitro*perinaphthindenone* (IVb) respectively. In higher concentration these products can be isolated in silky red crystalline needles, which may be identified by their melting points and mixed melting point determinations with authentic samples. The red compounds are similar to those prepared by Errera and Calderaro.

The reagents (IIa) and (IIb) are not only easily prepared, inexpensive and stable, but also have the following advantages:

(a) *Stability of the colour.* The characteristic red colour obtained is stable for about 24 hr.

(b) *Specificity of the reagent.* When solutions of *perinaphthindanetrione* hydrate or its 2-nitro derivative were added to solutions of some possible interfering substances in plant, animal and biological media, under the same experimental conditions used in the case of ascorbic acid (that is to say at room temperature) no colour was developed. The following substances were tested: glucose, fructose, alanine, leucine, isoleucine, phenylalanine, lactic acid, aceto-

Preparation and purification of dihydroxyperinaphthindenone (IVa) and its 2-nitro derivative (IVb). Ascorbic acid (Merck and Co. Inc.) (0.5 g.) in 5 ml. distilled water and 0.6 g. of pulverized *perinaphthindanetrione* hydrate dissolved in 20 ml. water are heated in a boiling-water bath for 3 min. and quickly cooled. The red silky crystalline precipitate is filtered off, washed several times with cold water and then dried; m.p. 258°, yield 0.42 g. This substance proved to be dihydroxyperinaphthindenone by melting point and mixed melting point with an authentic sample prepared

acetic acid, pyruvic acid, urea, uric acid, acetone and dehydroascorbic acid. On the other hand, reductones, reductic acid (Moubasher, 1948) and cysteine react at room temperature with *perinaphthindanetrione* hydrate or its 2-nitro derivative producing the same red colour as in the case of ascorbic acid. These substances, therefore, interfere with the estimation of ascorbic acid by our method.

METHODS AND RESULTS

Preparation and purification of perinaphthindanetrione hydrate (IIa). This compound is prepared according to Errera (1913). For purification, the trione is dissolved in hot ethanol and H₂S is then allowed to pass through the solution until complete precipitation of the crystalline red dihydroxyperinaphthindenone. The precipitate is filtered off, washed with a little hot ethanol, suspended in a small quantity of water and treated with hot saturated bromine water. This solution is filtered and the filtrate concentrated by boiling. If necessary, drops of bromine water are added to oxidize the small amounts of the dihydroxy derivative that may be formed during concentration. The concentrate is allowed to cool at room temperature. The almost colourless crystals obtained are repeatedly recrystallized from aqueous ethanol (1 vol. to 1 vol.). The crystals (m.p. 270°) are recovered by filtration and dried in a desiccator. The substance sublimes *in vacuo* forming intensely red crystals. It is soluble in water and in the majority of aqueous solvents, e.g. alcohols and acetic acid. It dissolves with difficulty in benzene, benzotrile, xylene and nitrobenzene at 100°, giving red solutions. It is practically insoluble in light petroleum (b.p. 50–60°) and dissolves in conc. H₂SO₄ forming an intense violet coloration which changes to orange after some time.

Preparation of 2-nitroperinaphthindanetrione hydrate (IIb). This compound is prepared according to the directions of Calderaro (1916). The substance (m.p. 173°) is purified by the procedure mentioned above. The principal properties of this compound are similar to those of the mother substance.

according to Errera. (Found: C, 73.6; H, 3.8. Calc. for C₁₃H₈O₃: C, 73.5; H, 3.7%). This substance is purified by repeated recrystallization from ethanol.

The dihydroxy-2-nitro derivative is prepared and purified by the procedure as above from 2-nitroperinaphthindanetrione hydrate. Deep-red needles, m.p. 265°, are obtained.

Spectrophotometric examination of perinaphthindanetrione hydrate, 2-nitroperinaphthindanetrione hydrate and their dihydroxy derivatives

The compounds used were pure products obtained by repeated recrystallization. All the photometric measurements recorded were made on a Hilger Barfit quartz spectrograph attached to a Spekker photometer, the light source being a condensed spark between tungsten steel. The solvent used in all cases was pure ethanol. Several concentrations of the purified substances and cells of different thicknesses were used. The spectroscopic data for the compounds are shown in Table 1 and graphically represented in Figs. 1 and 2. The absorption bands of *perinaphthindanetrione* hydrate and its 2-nitro derivative in ethanol have intensities which can be expressed as $E_{1\text{ cm}}^{1\%}$ at 320 mμ = 396 and $E_{1\text{ cm}}^{1\%}$ at 275 mμ = 626 for the two compounds respectively.

The dihydroxy derivatives of these two compounds have two distinct well separated maxima, one being in the ultraviolet and the other in the visible part of the spectrum.

Estimation of ascorbic acid

Ascorbic acid was estimated in pure solution, in urine and in blood plasma by three methods.

Spectrophotometric method. Ascorbic acid was estimated through spectrophotometric determination of dihydroxyperinaphthindenone produced by reduction of the reagent by

Table 1. Absorption spectra of perinaphthindanetrione hydrate, 2-nitroperinaphthindanetrione hydrate and their dihydroxy derivatives in ethanol

Substance	Wavelength of maxima (m μ .)	$E_{1\text{ cm.}}^{1\%}$	$E_{1\text{ cm.}}^{1\%}$ (mean value)	ϵ	log ϵ
periNaphthindanetrione hydrate	320	396, 391, 399, 396, 394, 399	396	9,029	3.95
2-Nitroperinaphthindanetrione hydrate	275 inflexion 335	611, 555, 671, 665	626	17,090	4.23
Dihydroxyperinaphthindenone	345	517, 532, 509, 533, 517, 555, 530, 515, 506	524	11,108	4.04
	475	150, 149, 151, 149, 153, 148	150	3,180	3.50
Dihydroxy-2-nitroperinaphthindenone	335	393, 375, 400, 393	390	10,023	4.00
	480	86, 83, 86, 85	85	2,184	3.34

ascorbic acid in pure solution, blood plasma filtrate and urine. The amount of the reduction product thus produced is estimated by the intensity of absorption at 475 m μ . (Table 1, Fig. 2); excess of the reagent, which should always be

parison with the above-mentioned methods. A standard curve was made according to instructions of the method, and was extended to cover a range from 0.1 to 300 μ g. ascorbic acid/ml.

Fig. 1. Absorption spectra of perinaphthindanetrione hydrate, —, and its 2-nitro derivative, ---, in ethanol.

present, does not absorb at this region of the spectrum. This is not the case if estimation is carried out at 345 m μ . (Table 1, Fig. 2) and higher values are obtained. However, good results have been obtained (absorption at 345 m μ .) if just enough reagent is added to react completely with the known amount of ascorbic acid present (in pure solution).

Photoelectric absorption method. A standard curve of dihydroxyperinaphthindenone in ethanol ranging from 1 to 30 mg./100 ml. was made with different amounts of the freshly crystallized product. The percentage transmission corresponding to these amounts was recorded with reference to ethanol set at 100% transmission. A Lumetron photoelectric apparatus with a blue-green glass filter (transmitting at 475 m μ .) was used.

Roe and Kuether's method. The method of Roe & Kuether (1943) for estimation of ascorbic acid was chosen for com-

Fig. 2. Absorption spectra of dihydroxy —, and dihydroxy-2-nitroperinaphthindenone ---, in ethanol.

Estimation of ascorbic acid in pure solution. To 5 ml. of freshly prepared standard ethanolic solution of ascorbic acid (11 mg./100 ml. ethanol) is added 1 ml. of freshly crystallized perinaphthindanetrione hydrate in ethanol (2 mg./ml.), mixed thoroughly and stoppered. The colour gradually develops and reaches its maximum after 10 min. The stability of the colour permits its measurement with ease any time from 10 min. to 24 hr. after the reagent has been added. The percentage transmission of the sample is recorded with reference to a blank tube of ethanol set at 100% transmission. The amount of dihydroxyperinaphthindenone formed is first estimated photoelectrically and then spectrophotometrically using a 5 mm. cell and the ascorbic acid present calculated from this.

Table 2 illustrates the results of ascorbic acid estimation in pure solution by the three methods.

Following the same procedure, ascorbic acid in ethanolic solution can be estimated by the derivative 2-nitroperinaphthindanetrione hydrate.

Estimation of ascorbic acid in urine. The urine of subjects living on a normal mixed diet without special preparatory period was slightly acidified just after voiding (10 ml. of 5% (w/v) trichloroacetic acid to 100 ml. urine). The acidified urine was well stirred and immediately filtered through a dry filter paper. Charcoal was not used to decolorize the urine, since this leads to some loss of ascorbic acid. To 5 ml. of this clear urine was added 1 ml. of a solution of perinaphthindanetrione hydrate 0.5 mg./ml. in water. This amount ensures complete reaction with ascorbic acid. Ethanol was not used because it causes the precipitation of inorganic salts present in urine. The solution was then mixed thoroughly, and after 10 min. the red colour of dihydroxyperinaphthindenone had completely developed and was ready for estimation. The control solution prepared by mixing thoroughly 5 ml. clear urine and 1 ml. water was put at 100% transmission and the percentage transmission of the treated urine recorded. This sample was then estimated spectrophotometrically using a 2 cm. cell.

Untreated urine and water as control shows no absorption at 475 m μ . The same sample of urine was estimated simultaneously by the method of Roe & Kuether. For recovery experiments (Table 3) known amounts of ascorbic acid in water were added to urine and then estimated by the three

methods. The table shows that the errors of estimation are about the same in all methods used.

Estimation of ascorbic acid in blood plasma. Blood was obtained by venepuncture from healthy individuals living on normal mixed diets. The oxalated blood was immediately centrifuged and the plasma separated. Plasma (10 ml.) was saturated with pure (NH₄)₂SO₄ crystals (8 g.) in a small beaker and well stirred. The mixture was then filtered with suction through a small Büchner funnel. The residue in the beaker was washed twice, with 1 ml. of 5% (w/v) metaphosphoric acid and the washings filtered. The filtrate was transferred quantitatively to a narrow finely graduated glass-stoppered measuring cylinder. About 3 ml. ethanolic solution of perinaphthindanetrione hydrate (0.4 mg./ml.) were added, the cylinder tightly stoppered and occasionally shaken in the course of half an hour.

Two layers separated: a lower colourless (NH₄)₂SO₄ layer and an upper red-coloured ethanolic layer (reduction product). The volume of the clear, coloured ethanolic layer was accurately measured and a sample portion was pipetted off and the colour estimated (ascorbic acid in this solution represents the amount of ascorbic acid in 10 ml. plasma). It has been found that there is no difference in the result if the control is pure ethanol or ethanolic extract of plasma treated exactly like the original sample. For simplicity ethanol can be used as control. Ascorbic acid in every sample of plasma was estimated by the three methods; for recovery experi-

Table 2. *Estimation of ascorbic acid in pure solution*

Ascorbic acid (mg./100 ml.)	Ascorbic acid found					
	Spectrophotometric method		Photoelectric method		Roe & Kuether's method	
	mg./100 ml.	Error (%)	mg./100 ml.	Error (%)	mg./100 ml.	Error (%)
30.0	29.22	-2.6	30.55	+1.8	29.0	-3.3
24.3	23.47	-3.4	23.96	-1.4	25.0	+2.9
20.7	19.92	-3.7	19.9	-3.7	21.4	+3.3
12.0	11.9	-0.83	12.21	+1.7	12.4	+3.3
10.0	9.81	-1.9	9.96	-0.4	10.0	0.0
5.1	4.98	-2.3	5.14	+0.8	5.2	+2.0
4.0	3.9	-2.5	4.0	0.0	4.1	+2.5
2.5	2.6	+4.0	2.52	+1.0	2.6	+4.0
1.5	1.56	+4.0	1.54	+2.6	1.48	-1.3
1.0	0.95	-5.0	0.98	-2.0	0.96	-4.0

Table 3. *Estimation of ascorbic acid in urine (recovery experiments)*

Method	Ascorbic acid, mg./100 ml.			Error of recovery (%)
	In normal urine	Amount added	Total found	
Spectrophotometric	2.98	0.97	3.98	+2.7
	1.99	0.64	2.65	+3.2
	1.65	0.35	1.99	-2.9
	3.31	0.35	3.65	-2.8
	2.65	1.00	3.6	-5.0
Photoelectric	3.03	0.97	4.03	+2.2
	2.09	0.64	2.74	+1.5
	1.6	0.35	1.94	-2.9
	3.28	0.35	3.63	0.0
	2.7	1.0	3.67	-3.0
Roe & Kuether (1943)	3.0	0.97	4.0	+3.0
	2.0	0.64	2.66	+3.1
	1.8	0.35	2.14	-2.8
	3.5	0.35	3.84	-2.8
	2.6	1.0	3.56	-4.0

Table 4. *Estimation of ascorbic acid in normal blood plasma (recovery experiments)*

Method	Ascorbic acid, mg. /100 ml.			Error of recovery (%)
	In normal plasma	Amount added	Total found	
Spectrophotometric	-0.79	0.5	1.28	-3.4
	-1.0	0.4	1.41	+2.5
	-1.0	1.0	1.96	-4.0
	-0.82	0.7	1.53	+1.4
	-0.82	1.2	2.18	-1.7
Photoelectric	-0.76	0.5	1.24	-4.0
	-1.03	0.4	1.43	+1.2
	-1.03	1.0	2.0	-3.0
	-0.84	0.7	1.56	+2.8
	-0.84	1.2	2.01	-2.5
Roe & Kuether (1943)	-0.72	0.5	1.2	-4.0
	-1.0	0.4	1.4	0.0
	-1.0	1.0	1.96	-4.0
	-0.88	0.7	1.6	+2.8
	-0.88	1.2	2.04	-3.3

ments (Table 4) known amounts of ascorbic acid were added to plasma which was treated in exactly the same way as normal plasma.

DISCUSSION

The advantages of the method described here are rapidity and simplicity. The reagent used is inexpensive and can easily be prepared and no special care need be taken regarding temperature or time of reaction. The possible interfering substances are reductones, reductic acid and cysteine. In Roe & Kuether's method hexoses, pentoses and glucuronic acid may interfere. Both methods seem to give about the same accuracy of result but the colorimetric method using *perinaphthindanetrione* is simpler and quicker.

SUMMARY

1. *perinaphthindanetrione* hydrate is used as a new reagent for the spectrographic estimation of ascorbic acid in pure solutions, blood plasma and urine.
2. The 2-nitro*perinaphthindanetrione* hydrate can also be used to estimate ascorbic acid in pure solutions.
3. This reagent is easily prepared, inexpensive, specific and stable.
4. The new method when compared with that of Roe & Kuether (1943) proved to be simpler and takes less time.

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The Inhibition of Histaminase by Amidines

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In a study of the inhibitory action of certain amidine derivatives on the enzyme amine oxidase it was found that the inhibition depended not only on the presence of the amidine group but on the configuration of the molecule as a whole (Blaschko & Duthie, 1945*a*). This study has recently been extended to a

number of isothioureia derivatives (Fastier & Hawkins, 1951).

Histaminase ('diamine oxidase') is also inhibited by some of these amidines (Blaschko & Duthie, 1944), and in the present paper we have examined the inhibitory action in more detail. The results show