The Use of *p*-Chloromercuribenzoic Acid in the Determination of Ascorbic Acid with 2:6-Dichlorophenolindophenol

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Methods based on the ability of ascorbic acid to reduce the dye 2:6-dichlorophenolindophenol (indophenol) to a colourless compound (Tillmans, Hirsch & Reinshagen, 1928) have been widely used in the estimation of ascorbic acid. Many other substances, including thiol compounds, thiosulphates, hydrogen sulphide, polyphenols and reductones (Lugg, 1942; Harris & Mapson, 1947) also decolorize indophenol and may therefore interfere with the determination of ascorbic acid. Most of these interfering substances reduce indophenol more slowly than does ascorbic acid (Harris & Mapson, 1947). The presence of substances which act in this way has been demonstrated in whole serum (Bierring, Mathiessen & Nielsen, 1945), and also in extracts of various mammalian tissues, e.g. kidney (Hopkins & Slater, 1935), brain (Young & Mitolo, 1934), whole blood (Butler & Cushman, 1940) and urine (Evelyn, Malloy & Rosen, 1938; Scarborough & Stewart, 1937). Mann & Leone (1953) reported that about 85% of the indophenol-reducing activity of hog semen was due to the presence of ergothioneine.

To increase the specificity of the indophenol method for the estimation of ascorbic acid Van Eekelen & Emmerie (1936) proposed the use of mercuric acetate to precipitate some of the interfering substances. An alternative method, based on the formation of complexes of interfering substances and formaldehyde, was proposed by Lugg (1942). However, methods based on these principles have certain disadvantages, which are discussed below.

In an attempt to devise a specific yet simple method for the determination of ascorbic acid in mammalian tissues we have found that *p*-chloromercuribenzoic acid successfully suppresses the interference due to thiol compounds. The results obtained by using this reagent in the determination of ascorbic acid in human plasma, erythrocytes and urine are presented. Preliminary communications of some of the results have already been given (Owen, Iggo & Horn, 1954; Owen & Iggo, 1955).

MATERIALS AND METHODS

Reagents. L-Dehydroascorbic acid was prepared by oxidation of ascorbic acid with quinone (Paterson, 1950). A stock solution of the sodium salt of 2:6-dichlorophenolindophenol (indophenol) containing 100 mg./100 ml. was made up weekly and kept at 5°. A stock solution [20% (w/v)] of metaphosphoric acid was made up at intervals of not longer than 4 days and kept at 5°. *p*-Chloromercuribenzoic acid (CMB) was prepared according to the method of Whitmore & Woodward (1946) and a solution containing 200 mg./100 ml. in 0·05N-NaOH was used. A solution of homocysteine was prepared by shaking for 30 min. 50 mg. of zinc dust in 20 ml. of a solution of homocysteine (L. Light and Co.), 68 mg./100 ml., in 2% (w/v) metaphosphoric acid and filtering off the excess of zinc. Titration against standard iodine solution showed that reduction was 90% complete. A solution of hydrogen sulphide was prepared by bubbling H_2S, generated by warming Sb_2S_5 in conc. HCl, into 2% (w/v) metaphosphoric acid. The concentration of hydrogen sulphide was determined by titration against $KMnO_4$.

Adsorption reagents: aluminium oxide for chromatographic analysis. Floridin earth, fuller's earth, light kaolin and acid-washed kieselguhr (all from British Drug Houses, Ltd.), Florisil (Floridin Co., Warren, Pa., U.S.A.), Celite (Johns Manville and Co. Ltd., London). Deactivated charcoal was prepared according to the method of Dalgliesh (1955).

Plasma. Protein-free filtrates of human plasma (oxalated or heparinized) were prepared by adding 2 vol. of plasma to 3 vol. of 3% (w/v) metaphosphoric acid.

Erythrocytes. Protein-free filtrates of human erythrocytes were prepared after preliminary treatment with carbon monoxide (Butler & Cushman, 1940). Whole blood (20 ml.) (oxalated or heparinized) was centrifuged and the leucocyte layer removed. The erythrocytes, resuspended in the plasma, were then placed in a tonometer (500 ml.), into which was led a steady stream of carbon monoxide generated from sodium formate and conc. H_2SO_4. The tonometer was rotated for 15 min. After centrifuging, 2 vol. of erythrocytes was added without delay to 3 vol. of 6% (w/v) metaphosphoric acid.

Urine. Fresh human urine, after filtration, was diluted to give a suitable indophenol-reducing activity. To 2 vol. of diluted urine was added 3 vol. of 3% (w/v) metaphosphoric acid.

Rat liver and kidney. The animals were killed by dislocation of the cervical vertebrae, and the organs dissected out and freed from any adhering fat without delay. Portions of the liver (approx. 3 g.) or the whole kidney (approx. 2 g.) were comminuted in 10 ml. of 6% (w/v) metaphosphoric acid in an all-glass tissue homogenizer. The extracts, made up to 15 ml., were allowed to stand for 15 min. and then filtered through two layers of Whatman no. 42 filter paper.

Measurement of pH. A Marconi pH meter (type TF S11D) was used.

Measurement of indophenol-reducing activity. The method used was similar to that of Evelyn *et al.* (1938). Into a

10 mm. cuvette was pipetted 2·0 ml. of the test solution followed by 0·5 ml. of sodium citrate (concentration according to final pH required) and 1·0 ml. of indophenol. After rapid mixing the optical density of the solution was measured with a Unicam SP. 600 spectrophotometer at 520 mμ. at 30 sec., or stated intervals, after the addition of indophenol. Allowance was made for any turbidity or colour in tissue extracts by measuring the optical density of the solution immediately after decolorization of the remaining indophenol with a slight excess of ascorbic acid. Calibration curves were prepared for each experiment with standard solutions of ascorbic acid in 1·8% (w/v) metaphosphoric acid buffered to the approximate pH.

Reaction with p-*chloromercuribenzoic acid* (*CMB*). To 3 vol. of the test solution was added 1 vol. of CMB. After standing for 5–10 min. a precipitate, consisting of excess of reagent together with any insoluble complex formed by interaction with substances present, was removed by centrifuging and the supernatant solution used for colorimetry. CMB is only sparingly soluble in aqueous solutions at pH 2–5, and the concentration of CMB in the supernatant was not determined.

RESULTS

The stability of indophenol at low pH was examined by measuring the rate of decrease in optical density of dye in metaphosphoric acid solutions buffered with sodium citrate (Fig. 1). Below pH 3·5 appreciable fading occurred within 5 min. of the addition of the dye. The rate of fading was that of a first-order reaction. At pH 5·0 the solution was purple, indicating that some of the dye was in the anionic form.

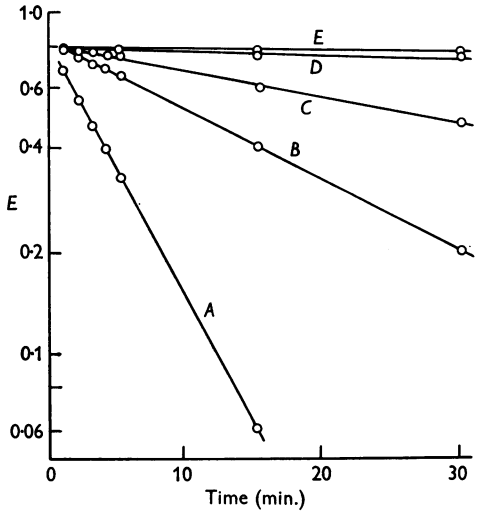

Fig. 1. Effect of pH on the stability of indophenol. Each solution comprises 2·0 ml. of 2% (w/v) metaphosphoric acid buffered with sodium citrate + 1·0 ml. of indophenol (10 mg./100 ml.). pH: *A*, 2·0; *B*, 2·4; *C*, 2·9; *D*, 3·5; *E*, 4·2.

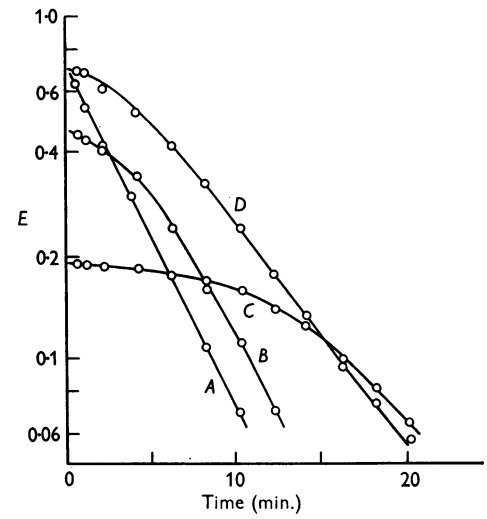

Fig. 2. Effect of added substances on the stability of indophenol at pH 1·8. *A*, 2·0 ml. of 2% (w/v) metaphosphoric acid + 0·5 ml. of H_2O + 1·0 ml. of indophenol (10 mg./100 ml.); *B*, as *A* but with metaphosphoric acid containing ascorbic acid, 1 mg./100 ml.; *C*, as *A* but with metaphosphoric acid containing ascorbic acid, 2 mg./100 ml.; *D*, as *A* but with 0·5 ml. of reduced dye solution (125 μg. of reduced dye) instead of 0·5 ml. of H_2O.

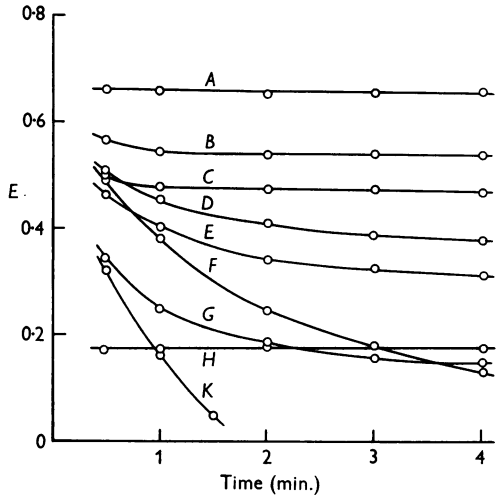

Fig. 3. Rates of reaction of various substances with indophenol at pH 4·5. Each solution comprises 2·0 ml. of 2% (w/v) metaphosphoric acid + 0·5 ml. of 12% (w/v) sodium citrate + 1·0 ml. of indophenol (10 mg./100 ml.) with the following additions: *A*, none; *B*, thiourea, 5×10^{-4}M; *C*, $Na_2S_2O_3$, $0{\cdot}6 \times 10^{-4}$M; *D*, H_2S, 5×10^{-4}M; *E*, Na_2SO_3, $2{\cdot}5 \times 10^{-4}$M; *F*, glutathione, 5×10^{-4}M; *G*, thioglycollic acid, 2×10^{-4}M; *H*, ascorbic acid, 1×10^{-4}M; *K*, cysteine, 5×10^{-4}M.

Partial decolorization of the dye with ascorbic acid leads to a decrease in the rate of fading which occurs spontaneously at pH about 2·0 (Mindlin & Butler, 1937–8; Lugg, 1942; Stewart, Horn & Robson, 1953). Mindlin & Butler reported, further, that the addition of dehydroascorbic acid did not affect the rate of fading, whereas the addition of reduced (leuco) dye stabilized the dye. We have found, however, that the stabilization of residual dye after the addition of ascorbic acid is incomplete and temporary (Fig. 2). Addition of reduced dye prepared by reaction of dye with zinc dust, or with ascorbic acid, also increased the stability of the dye temporarily. Dehydroascorbic acid had no stabilizing effect.

The reaction of various substances with indophenol was examined by measuring the rate of decolorization of the dye at pH 3·5 and 4·5. Ascorbic acid, thiosulphate and thiourea reacted relatively quickly, so that an equilibrium was reached within 60 sec. (Fig. 3). With the other substances tested an equilibrium was not reached within 4 min. Of the compounds examined all reacted with indophenol more rapidly at the higher pH except thiosulphate, thiourea and ergothioneine, which reduced indophenol more rapidly at the lower pH (Table 1).

CMB did not affect the ability of ascorbic acid to reduce indophenol, but inhibited almost completely the indophenol-reducing activity of all the other compounds tested with the exception of sulphite. This inhibition persisted for at least 5 min. (Table 1). It was found, further, that CMB inhibited the indophenol-reducing activity of glutathione, cysteine, hydrogen sulphide or thiosulphate, when added to metaphosphoric acid extracts of human plasma, erythrocytes, urine or of rat liver, but it did not affect the recovery of ascorbic acid added to these extracts (Table 2).

The effect of CMB on the indophenol-reducing activity (apparent ascorbic acid content) of human plasma, erythrocytes and urine was then

Table 1. *Indophenol-reducing activity of various compounds*

Results are expressed in terms of equivalent ascorbic acid concentration (10^{-5} M).

		No addition						With CMB (50 mg./100 ml.)
		30 sec.		1 min.		5 min.		5 min.
Compound	Concn. (10^{-5} M)	pH 4·5	pH 3·5	pH 4·5	pH 3·5	pH 4·5	pH 3·5	pH 3·5
Ascorbic acid	5	5·0	5·0	5·0	5·0	5·0	5·0	5·0
Sodium thiosulphate	6	3·6	4·1	3·6	4·0	4·1	3·6	0·0
Sodium sulphite	25	5·0	4·8	5·5	5·5	8·4	7·7	2·7
Cysteine	50	7·1	2·6	10·3	6·8	> 20	> 20	0·0
2:3-Dimercaptopropanol	50	6·8	5·1	9·8	7·5	13·5	11·5	0·1
Ergothioneine	50	0·0	2·7	0·0	4·2	0·0	9·6	0·0
Glutathione	50	3·3	0·7	5·8	1·3	11·7	6·3	0·0
Hydrogen sulphide	50	3·1	2·6	4·4	3·2	6·1	4·8	0·2
Homocysteine	50	0·7	0·3	1·3	0·4	6·4	1·6	0·1
Thiourea	150	2·0	3·2	2·5	3·6	2·5	7·0	0·0

Table 2. *Effect of* p-*chloromercuribenzoic acid on the indophenol-reducing activity of compounds added to biological material*

Substances were added in amounts calculated to give approximately equal increments in indophenol-reducing activity. Results in terms of ascorbic acid (μg./100 ml.). Values in parentheses give percentage of added ascorbic acid recovered.

	Addition					
Material	None	Ascorbic acid (400 μg./100 ml.)	Glutathione (12 mg./100 ml.)	Cysteine (5 mg./100 ml.)	H_2S (1·4 mg./100 ml.)	Thiosulphate (400 μg./100 ml.)
Plasma filtrates	444	868 (105)	448	448	444	448
	404	832 (107)	404	424	408	416
	424	840 (104)	448	448	424	416
Erythrocyte filtrates	232	—	240	244	252	232
	212	636 (106)	232	196	200	216
	312	684 (93)	308	—	288	300
Diluted urine	316	688 (93)	308	316	300	308
	400	796 (99)	400	400	400	432
	272	664 (98)	264	232	256	256
Diluted liver filtrate	452	872 (105)	452	452	452	456

examined (Fig. 4). Added to filtered metaphosphoric acid extracts of plasma this compound produced a negligible effect on the apparent ascorbic acid content in filtered extracts of plasma. In contrast to extracts of plasma, metaphosphoric acid extracts of erythrocytes contained appreciable quantities of slow-reacting material. The effect of this was noticeably greater at pH 4·5 than at pH 3·5. The addition of CMB produced a decrease in the apparent ascorbic acid content and progressive decolorization of the dye no longer occurred.

Human urine also showed the presence of slow-reacting substances (Fig. 4). The effect of these, however, was only partly abolished by CMB. Further treatment of the extracts with the adsorption reagents alumina, Celite, Floridin earth, Florisil, fuller's earth, kaolin and kieselguhr failed to remove all the remaining slow-reacting material. Deactivated charcoal removed most of the interfering substances, but at the same time destroyed most of the ascorbic acid.

The quantitative effect of CMB is shown in Table 3. The mean decrease in the apparent ascorbic acid content of plasma was about 2%, of erythrocytes about 50%, and of urine about 15%. In these analyses the reduction of indophenol was measured at pH within the range 3·6–4·4.

In a few experiments the indophenol-reducing activity of filtered metaphosphoric acid extracts of rat liver and kidney was examined. Filtrates of both these tissues contained appreciable quantities of interfering material (Fig. 5), the effect of which was removed almost completely by CMB.

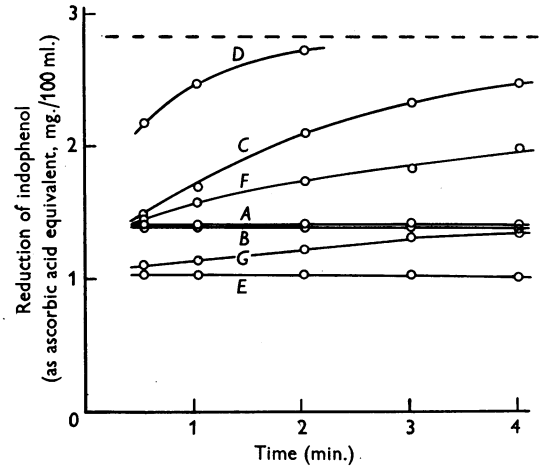

Fig. 4. Effect of *p*-chloromercuribenzoic acid (CMB) on the indophenol-reducing activity of human plasma, erythrocytes and urine. Each solution comprises 2·0 ml. of test solution + 0·5 ml. of sodium citrate + 1·0 ml. of indophenol (4 mg./100 ml.). Test solutions (pH values are those of reaction mixture): *A*, plasma filtrates, pH 3·5; *B*, as *A* + CMB; *C*, erythrocyte filtrates, pH 3·5; *D*, as *C*, pH 4·5; *E*, as *C* + CMB; *F*, diluted urine, pH 3·5; *G*, as *F* + CMB. Amount of CMB added, 50 mg./100 ml. Interrupted horizontal line corresponds to complete decolorization of dye.

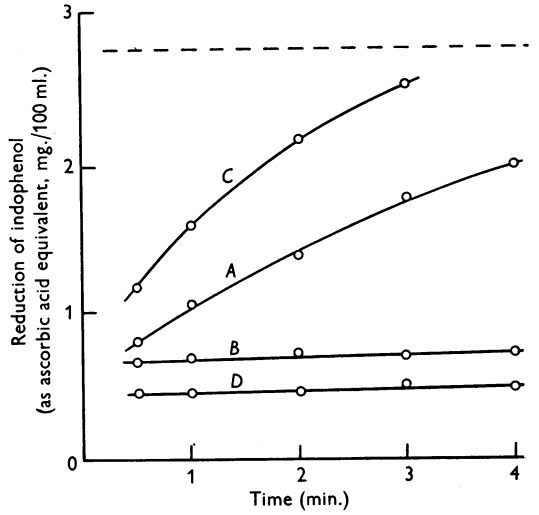

Fig. 5. Effect of *p*-chloromercuribenzoic acid (CMB) on indophenol-reducing activity of rat liver and kidney. Each solution comprises 2·0 ml. of test solution + 1·0 ml. of indophenol (4 mg./100 ml.). Test solutions: *A*, liver filtrate, diluted 1/4, buffered with sodium citrate (≡ 7·5 g. of wet tissue/100 ml.); *B*, as *A* + CMB; *C*, kidney filtrate buffered with sodium citrate (≡ 20 g. of wet tissue/100 ml.); *D*, as *C* + CMB. Amount of CMB added, 50 mg./100 ml. Interrupted horizontal line corresponds to complete decolorization of dye.

Table 3. *Effect of CMB on apparent ascorbic acid content of plasma, erythrocytes and urine*

Results are given as means (with ranges in parentheses) in terms of ascorbic acid (mg./100 ml.). The amount of CMB added was 50 mg./100 ml.

Material	No. of expts.	Without CMB	With CMB	Differences
Plasma	40	0·56 (0·09 to 1·40)	0·55 (0·10 to 1·40)	0·01 (−0·09 to +0·07)
Erythrocytes	12	0·47 (0·21 to 1·39)	0·25 (0·10 to 0·94)	0·22 (+0·01 to +0·45)
Urine (diluted)	12	0·77 (0·19 to 1·92)	0·65 (0·02 to 1·79)	0·12 (0·00 to +0·26)

DISCUSSION

To increase the specificity of the indophenol method for the determination of ascorbic acid in biological material, Van Eekelen & Emmerie (1936) proposed treatment of extracts with mercuric acetate to remove thiol compounds and other interfering substances. Subsequently, the extracts were treated with hydrogen sulphide to remove the excess of mercuric acetate and to reduce any dehydroascorbic acid formed by the reaction of ascorbic acid with Hg^{2+} ions. This procedure, however, does not permit the determination of ascorbic acid in the presence of dehydroascorbic acid. Furthermore, several workers have commented on the difficulty of removing, from the extracts, all traces of hydrogen sulphide before measurement of the indophenol-reducing activity, and have drawn attention to the possibility that treatment of tissue extracts with hydrogen sulphide may produce interfering substances not present originally (Johnson, 1933; Mack & Tresler, 1937; King, 1936; Kellie & Zilva, 1936; Bessey, 1938). This has been shown to occur when certain aldehydes, ketones or quinones are treated with hydrogen sulphide (Lugg, 1942; King, 1941; Smythe & King, 1942; Hochberg, Melnick & Oser, 1943). In this connexion Kellie & Zilva (1936) concluded from analyses of ultraviolet absorption spectra that the increase in the indophenol-reducing activity of filtrates of guinea-pig plasma treated with hydrogen sulphide was due to the formation of reducing substances other than ascorbic acid. The presence of increased amounts of interfering substances has also been reported after treatment with hydrogen sulphide of whole-blood filtrates (Butler & Cushman, 1940), and of urine (Evelyn *et al.* 1938; Scarborough & Stewart, 1937).

In contrast, the proposed use of *p*-chloromercuribenzoic acid avoids the errors which may be introduced by the use of hydrogen sulphide and also permits the estimation of ascorbic acid in the presence of dehydroascorbic acid. The action of CMB is presumably due to its powerful tendency to form complexes with thiol groups (Barron & Singer, 1945) so that the reducing ability of the latter is suppressed. However, our findings indicate that it reacts also with thiosulphate and to some extent with sulphite. The nature of the latter two reactions is, at present, not clear.

In view of the relative rapidity of the reduction of indophenol by ascorbic acid, measurement of the amount of indophenol reduced at intervals and extrapolation of these values, plotted against time, to zero time, have been proposed as a means of obviating interference due to slow-reacting substances (Evelyn *et al.* 1938). It is obvious from Fig. 3, however, that for most of the interfering substances a curvilinear extrapolation would be necessary to give zero indophenol reduction at zero time. The inherent difficulties in curvilinear extrapolation make this procedure impracticable.

As a new approach to the problem of non-specific reduction of indophenol Lugg (1942) proposed the use of formaldehyde which condenses to an extent depending on the pH with many interfering substances, and also with ascorbic acid, to form feebly reducing or non-reducing complexes. Of these substances which have been examined only ascorbic acid, reductones and a hydroxytetronic acid (the lactone of 1:2(?):3-trihydroxyprop-1-ene-1-carboxylic acid) condense slowly at pH 1·5 and rapidly at pH 3·5. Thus in the absence of the last-named compounds the difference in indophenol-reducing activities after condensation at pH 1·5 and 3·5 respectively may be taken as a measure of the ascorbic acid present. Methods based on this principle have been applied to the estimation of ascorbic acid in urine (Mapson, 1953; McSwiney, Clayton & Prunty, 1954). However, this procedure is laborious (Harris & Mapson, 1947) and requires careful control of conditions (Snow & Zilva, 1944). We have found, in agreement with others (McSwiney *et al.* 1954), that this procedure cannot accurately be applied to materials in which the concentration of ascorbic acid is low, i.e. less than 1·0 mg./100 ml. Nevertheless, in normal urine, where the concentration of ascorbic acid is relatively high, the formaldehyde method may provide a more accurate result than that given by the method described in this paper, since the result obtained by the latter applied to urine includes some reducing material other than ascorbic acid.

The negligible effect of CMB on the indophenol-reducing activity of plasma is consistent with the absence from plasma extracts of substances reacting slowly with indophenol. The interfering material in human erythrocytes presumably includes glutathione and ergothioneine. The absence of progressive decolorization of indophenol after the addition of CMB to extracts of plasma or erythrocytes suggests that the results represent the true ascorbic acid content, though the presence of substances other than ascorbic acid, which react rapidly with indophenol and which are not removed by CMB, is not excluded. Examination of the reduction of indophenol by these filtrates by the 'continuous-flow' method devised by Harris & Mapson (1947) might enable this point to be settled.

Using formaldehyde, Mapson (1953) showed the presence of two groups of interfering substances in urine. The first group contains substances which form non-reducing complexes rapidly at pH 0·6; at this pH ascorbic acid condenses only slowly.

Any thiol compounds, sulphides and thiosulphates present are included in this group, and it seems reasonable to conclude that this group corresponds roughly to the material removed by CMB. The interfering substances in the second group condense only slowly both at pH 0·6 and at pH 3·5, whereas ascorbic acid itself condenses rapidly at this latter pH. It is concluded that the interfering material remaining after treatment of urine extracts with CMB corresponds roughly to this second group of substances and also to the interfering material not removed by treatment with mercuric acetate (Evelyn *et al.* 1938). The nature of this material remains unknown. It will include any polyphenols present (Lugg, 1942).

The instability of indophenol at low pH is characteristic of indophenol dyes (Karrer, 1950), the latter decomposing to give quinones and aminophenols. The partial stabilization of indophenol by reduced (leuco) dye may be due to the formation of a reversible complex of oxidized and reduced dye, possibly analogous to quinhydrone. The stabilization, however, is not sufficient to enable the reduction of indophenol to be measured satisfactorily at pH 3·0, as has been advocated (Bessey, 1938; Harris & Olliver, 1942) to lessen interference due to other reducing substances. Indeed, the interference due to some compounds, e.g. thiosulphate and ergothioneine, is greater at lower pH.

SUMMARY

1. The stability of indophenol at low pH has been examined. The amount of spontaneous fading which occurs below pH 3·5 makes the colorimetric estimation of indophenol-reducing activity impracticable in solutions of pH less than this value.

2. The reaction of various reducing substances with indophenol and the interference which their presence would cause in the estimation of ascorbic acid has been examined.

3. Added *p*-chloromercuribenzoic acid (CMB) does not affect the reaction between ascorbic acid and indophenol, but it suppresses almost completely the decolorization of indophenol by a number of interfering substances.

4. Metaphosphoric extracts of human plasma contain only negligible quantities of interfering material removed by CMB, whereas extracts of human erythrocytes, rat liver and kidney contain appreciable amounts of interfering material which are almost completely removed by this compound. Human urine contains interfering material, some of which is removed by CMB.

5. It is concluded that *p*-chloromercuribenzoic acid may be used to increase the specificity of the indophenol method for the determination of ascorbic acid in biological material.

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