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A Critical Examination of Lugg's Method for the Determination of *l*-Ascorbic Acid. 2

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Our previous contribution to the subject (Snow & Zilva, 1943) was devoted mainly to the study of the kinetics of the combination of pure ascorbic acid, reductic acid and reductone with HCHO, and the results were utilized for the consideration of the limitations of Lugg's method (1942). Further observations made us aware of the fact that certain indophenol-reducing substances which are formed on heating solutions containing glucose in the presence of alkali behaved differently in their combination with HCHO to pure reductone. It therefore became desirable to investigate in detail the problem that arose. The results which are reported in this communication, apart from raising points of theoretical interest, throw further light on the limitations of the method. A technique which we consider yields the most satisfactory results under the circumstances is described.

METHODS

Reagents

l-Ascorbic acid and reductic acid (see Snow & Zilva, 1941).

Reductone (see Snow & Zilva, 1938).

Formaldehyde (see Snow & Zilva, 1943).

Metaphosphoric acid. Laboratory reagent. Solutions prepared freshly each day.

Pectin. Commercial specimen prepared from lemons. Completely soluble in water, giving an almost colourless opalescent solution. pH of 1% w/v solution = 3.04. Free acid—equivalent to 0.98 ml. *N*-NaOH/g. (indicator—phenolphthalein).

Glycine. Anal. reagent twice recrystallized from water.

Ethylaminoacetate hydrochloride. Prepared from Anal. glycine and recrystallized from ethanol; m.p. 143°.

Methyleneaminocetonitrile. Dimeric form prepared by the method of Klages (1903); twice recrystallized from ethanol; m.p. 129°. The molar concentrations of this com-

pound given in this paper are calculated on the basis of the monomeric formula.

Methylamine. Dilute solutions prepared from a 33% aqueous solution. Concentration determined by titration with HCl.

Alanine (dl). Synthetic commercial specimen.

Glutamic acid. Commercial specimen containing a mixture of the enantiomorphs.

Other compounds employed were all Anal. reagents.

Indophenol titration. Solutions containing ascorbic acid or reductic acid were titrated directly with *mn*-2,6-dichlorophenolindophenol. With reductone or alkali-treated glucose solutions, however, direct titration was unsatisfactory owing to the slowness of the reaction. In these cases the reducing solution was run into an excess of acidified indophenol solution, allowed to react for 1 min., and the excess of the indicator back-titrated with stabilized *mn*-ascorbic acid solution.

Treatment of glucose solutions with varying concentrations of sodium hydroxide. 20 ml. 2.5% w/v glucose solution together with sufficient water to give a total volume of 25 ml. after the addition of the necessary quantity of NaOH were placed in a boiling tube in a water-bath at 91.5° so that the temperature inside the tube was 90°. After the addition of NaOH the solutions were mixed for a short time by a current of nitrogen; continuous bubbling of the gas was undesirable owing to evaporation. At the end of the required period the reaction was stopped by the addition of a small excess of HCl. The solutions were cooled in an atmosphere of nitrogen, diluted to a known volume, and the reducing value assessed by indophenol titration.

Buffers

For adjustment to ...	pH 3.5	pH 1.5
ml. 10% $\text{CCl}_3\text{CO}_2\text{H}$	417	417
g. NaCl	167	167
g. KH_2PO_4	23.3	23.3
g. citric acid	36	36
ml. 4 <i>M</i> -sodium acetate	123	30.4
Water	To 1 l.	To 1 l.

For convenience these solutions are referred to below as 'chloride-citrate buffers'.

For experiments at pH 2.0: 150 ml. 8% w/v metaphosphoric acid solution, 31.8 ml. M-secondary sodium citrate solution, water to 200 ml.

Reaction of reducing compounds with formaldehyde at different pH values. The reaction mixtures were made up as follows:

	pH 3.5	pH 1.5	pH 2.0
ml. stock buffer solution	20	20	20
ml. aqueous solution of added substances	20	24	20
ml. 12M-formaldehyde solution	2	2	10
Concentration of HCHO in the reaction mixture (M)	0.57	0.52	2.4

The pH of the reaction mixture was always checked by means of a glass electrode and adjustments made if necessary by the addition of HCl or sodium acetate solution.

The solutions containing approximately 0.9 mM reducing substance were allowed to attain the temperature of the thermostat (usually 25°). The HCHO solution was added at a given time; portions were subsequently withdrawn at intervals, acidified to stop the reaction and titrated with indophenol. Pure nitrogen was passed through the solutions during the whole experiment to prevent atmospheric oxidation. The initial titration value of the reducing compound was determined on a separate solution in which the HCHO was replaced by water.

Oxidation and reduction of alkali-treated glucose solutions. A suitable volume of the reducing solution was added to

25 ml. citrate buffer (to give a pH of 3.5 after dilution) and diluted to approximately 40 ml. A few drops of starch solution were added and the solution then titrated with 0.1 N iodine until a permanent blue colour appeared. The volume was made up to 50 ml., H₂S was passed immediately through the oxidized solutions for 5 min. and the vessel then sealed and allowed to stand for the stated time. At the end of this period nitrogen was passed through the solution to remove the excess of H₂S. The complete removal of H₂S was ensured by testing the issuing gas with dry lead acetate paper for 5 min. The reducing value of the solutions was then assessed by titration with mN-indophenol.

The action of acid upon pectin solutions. 10 ml. of the solutions containing 1% w/v citrous pectin and the required concentration of acid were introduced into pyrex tubes and sealed. The ampoules were then heated in a boiling water-bath or in an oil-bath maintained at 120° for the required time, rapidly cooled and a sample titrated with indophenol.

RESULTS

Reducing power of solutions prepared by the treatment of glucose with alkali under varying conditions

The only record in the literature, as far as we are aware, of a quantitative experiment upon the indophenol-reducing capacity of glucose solutions treated with alkali is that of Kertesz (1934), who employed only one concentration of alkali. To clarify our problem, a 2% w/v glucose solution was heated at 90° with different concentrations of NaOH for varying times. The results of these experiments are

Fig. 1. The reducing value of solutions obtained by the treatment of glucose with varying concentrations of NaOH. Conditions of preparation: temp. 90°; glucose concentration 2% w/v. The figures against the curves indicate the concentration of NaOH (N). In the right-hand curve maximum reducing values are plotted against NaOH concentration.

represented graphically in Fig. 1, the reducing values being in terms of normality. It will be seen that at low concentrations of alkali the reducing value reached a maximum after about 6 min. and then remained almost constant. At higher NaOH concentrations the maximum was reached even earlier. In this case there was, in contrast to the lower concentrations, a slow diminution in the reducing value after the maximum had been reached. The maximum reducing value at low concentrations of alkali was directly proportional to the normality of the NaOH. With increasing concentrations, however, the maximum reducing power first increased more slowly and finally decreased. There was thus a particular combination of conditions under which the greatest possible concentration of reducing material could be obtained. This maximum occurred when the molar concentration of NaOH was about twice that of the glucose.

The action of formaldehyde on alkali-treated glucose solutions

The above experiments yielded data concerning glucose solutions heated for various times in the presence of various concentrations of alkali, and a range of such solutions differing from each other widely in the conditions of preparation were chosen for the investigation of their reaction with HCHO at pH 3.5. It would appear from Fig. 2 that the

Fig. 2. The reaction of formaldehyde at pH 3.5 with alkali-treated glucose solutions prepared under different conditions. The figures against the curves refer to the alkali-treated glucose solutions as described in Table 2. Chloride-citrate buffer. Initial concentration of reducing substances approx. 0.9M. HCHO concentration 0.57M. Temp. 25°.

conditions of preparation greatly influenced the course of the reaction with HCHO. The curve for solution 1 indicated a moderately rapid primary reaction succeeded by a much slower secondary reaction represented by an almost linear portion of the graph. At the other extreme the initial reaction

with solution 4 was considerably more rapid. Although the rate of this reaction steadily declined there did not appear to be any evidence within the duration of the experiment of a succeeding linear reaction similar to that observed with solution 1. Curves for the other three solutions were intermediate in their characteristics. The striking fact is that none of the curves was similar in form to that obtained with purified reductone (see Fig. 3). Consequently the correction given for use with purified

Fig. 3. Reaction of formaldehyde with reducing solutions at pH 3.5 and 2.0. A. Alkali-treated glucose solution 1 (see Table 2). B. Alkali-treated glucose solution 4 (see Table 2). C. Purified reductone. D. Ascorbic acid. Initial concentration of reducing solutions approx. 0.9M. Temp. 25°. pH 3.5 experiments. Chloride-citrate buffer. HCHO concentration 0.57M. Time scale 1. pH 2.0 experiments. Metaphosphate-citrate buffer. HCHO concentration 2.4M. Time scale 2.

reductone (Snow & Zilva, 1943) cannot be applied successfully to alkali-treated glucose solutions because the course of the reaction is not linear in its initial stages. The failure of this type of correction applies also to the extrapolation recommended by Mapson (1943a,b).

The latter writer claims that, although the reaction at pH 3.5 is unsatisfactory, accurate results may be obtained by working at pH 2.0. We considered it necessary to ascertain the validity of this claim by comparing the behaviour of certain reducing solutions at pH 3.5 under our conditions with the behaviour of the same solutions at pH 2.0 under Mapson's conditions. The solutions chosen were ascorbic acid, purified reductone and alkali-

treated glucose solutions 1 and 4 (cf. Fig. 2), i.e. those showing the most extreme differences of behaviour. It was found that with ascorbic acid the velocity of reaction at pH 3.5 was almost exactly six times that at pH 2.0. In Fig. 3 the comparative results are given; the time scale for the pH 3.5 experiments is six times that for experiments at

fraction of the reducing material had combined at the point where the curve became linear. It is thus seen that these results do not uphold Mapson's assertion that the course of the reaction at pH 3.5 differs fundamentally from that at pH 2.0. Therefore no advantage is gained by carrying out the reaction at pH 2.0:

Another point of importance was to ascertain whether the course of the HCHO reaction with mixtures of different reducing compounds was equivalent to the sum of the reactions of the individual components. A typical experiment is illustrated in Fig. 4. In every case the observed results approximated very closely to the course of reaction calculated by summation of the reactions of the individual compounds. When the linear part of the curve (A) obtained with the alkali-treated glucose solution was extrapolated to zero time, the figure obtained corresponded to 13% of the initial reducing value. In a mixture of the same reducing solution with a known quantity of ascorbic acid, approximately the same percentage of the reducing value remained after extrapolation. Thus in the case of curve C, in which 42% of the total reducing value was due to pure ascorbic acid, extrapolation gave an 'ascorbic acid value' of 51.5%. When curve D was extrapolated, the 'ascorbic acid value' of the solution obtained was 50% of the total reducing value, a figure which agreed well with the proportion of ascorbic acid actually present (51%). This result would be expected with purified reductone where the course of the reaction is linear in its initial stages. It may therefore be stated that when purified reductone is present, the error in Lugg's method is

Fig. 4. The reaction of formaldehyde with mixed reducing substances at pH 3.5.

Curve	Reducing substances	Symbols	
		Observed values	Values calculated by summation
A	Alkali-treated glucose solution (100)	○	—
B	Alkali-treated glucose solution (47) + reductone (53)	△	▽
C	Alkali-treated glucose solution (58) + ascorbic acid (42)	⊙	□
D	Reductone (49) + ascorbic acid (51)	◇	×

The figures in brackets indicate the percentage of the total initial reducing value contributed by each component. Corresponding curves for reductone and ascorbic acid alone are given in Fig. 3 to avoid repetition. Chloride-citrate buffer. Total initial concentration of reducing substances approx. 0.9mn. HCHO concentration 0.57M. Temp. 25°.

pH 2.0. It will be seen that the courses of the reactions at pH 2.0 were qualitatively very similar to those at pH 3.5. In the case of the purified reductone and solution 4 the velocity of the reaction compared with ascorbic acid was relatively slower at pH 2.0. With solution 1, however, at the lower pH the relative velocity was greater, and a greater

Table 1. Errors in the estimation of ascorbic acid by Lugg's method in the presence of reductone or of alkali-treated glucose solutions (calculated from Fig. 3)

Reducing solution	pH of HCHO reaction	% of total reducing value obtained as 'ascorbic acid'		
		Lugg's technique uncorrected	Corrected by formula	Corrected by extrapolation
Purified reductone	3.5	30	5	—
Purified reductone	2.0	22	7	—
Alkali-treated glucose solution 1	3.5	13	8	15
Alkali-treated glucose solution 1	2.0	20	15	22
Alkali-treated glucose solution 4	3.5	31	17	—*
Alkali-treated glucose solution 4	2.0	31	22	33

* No extrapolation was possible in this case because the curve did not become linear.

small whether corrected by extrapolation or by the application of our formula (vide Snow & Zilva, 1943). In the case of alkali-treated glucose solutions, however, a considerable error may be expected, the magnitude of which will vary according to the method of preparation of the reducing solution and which is thus unpredictable. An indication of the order of the error that may occur is given by the figures in Table 1. These results show the danger of the random application of Lugg's method, even when corrections are employed.

The differential behaviour of purified reductone and of alkali-treated glucose solutions after oxidation, and reduction with H_2S

In our previous paper we drew attention to the fact that when solutions of purified reductone are oxidized and subsequently reduced with H_2S at pH 3.5 only a small fraction of the original reducing value is regenerated. Mapson (1943*b*) found, on the other hand, that by reducing alkali-treated glucose solutions oxidized with iodine with H_2S for 5 hr., the entire reducing capacity could be regenerated. This suggested a further difference between the chemical behaviour of purified reductone and that of alkali-treated glucose solutions. We have therefore investigated the regeneration by H_2S reduction, after oxidation with iodine, of the reducing capacity of the alkali-treated glucose solutions used in the experiments of the preceding section. The results which are given in Table 2 show considerable differences between the various solutions. With solution 1

Table 2. Recovery of reducing capacity from oxidized alkali-treated glucose solutions after treatment with H_2S

Solution no.	Conditions of preparation		% of original reducing capacity recovered after oxidation and subsequent treatment with H_2S for (hr.)		
	NaOH concentration (N)	Time of heating (min.)			
			1	5	20
1	0.02	10	93	100	107
2	0.15	5	76	83	87
3	0.3	5	71	75	83
4	0.3	0.5	55	62	75
5	0.85	15	82	85	93

the reduction with H_2S was very rapid and was complete at the end of 5 hr.; longer periods tended to lead to a reducing value in excess of that originally present. At the other extreme, solution 4 even after 20 hr. reduction showed only a 75% recovery. With purified reductone it has been found that the recovery was extremely variable, but in general the figures were low and varied from 5 to 15%. When Table 2 and Fig. 2 are examined it will be seen that the alkali-treated glucose solutions may be arranged

in a series showing progressive changes of behaviour in both types of reaction. The order of the solutions in the series is the same in both cases.

The reducing power of solutions prepared by the treatment of pectin with acid under varying conditions

On theoretical grounds it would be expected that under most conditions prevailing in the preparation of food, reductic acid is more likely to be formed than reductone. It was therefore decided to investigate in detail the conditions under which reductic acid is formed from citrous pectin, a precursor of reductic acid. The pectin was heated for various periods of time in the presence of different concentrations of acid.

From the results given in Fig. 5 it may be assumed that little reductic acid would be formed during the preparation of foods at a temperature of 100° , since

Fig. 5. The production of reductic acid from pectin by the action of acid under varying conditions.

Concentration of citrous pectin = 1% w/v.

Temp.	Acid	Curve
120°	2N- H_2SO_4	A
120°	0.5N- H_2SO_4	B
120°	0.25M-citric acid	C
120°	No addition	D
100°	2N- H_2SO_4	E
100°	0.5N- H_2SO_4	F
100°	0.25M-citric acid	No detectable reducing value was obtained after 6 hr.
100°	No addition	

in the absence of acid or in the presence of 0.25M-citric acid no reducing substances were formed after 6 hr. Even in the presence of 0.5N- H_2SO_4 the

Table 3. *The reaction between formaldehyde and reductic acid or acid-treated pectin solution at pH 3.5*

Chloride-citrate buffer. Initial concentration of reducing substances approx. 0.9mM. Concentration of HCHO 0.57M. Temp. 25°.

Reducing solution	Percentage of initial reducing value remaining after (min.)										
	$\frac{1}{2}$	1	2	5	10	15	30	45	60	75	90
Reductic acid	95.4	94.6	94.6	92.6	91.8	90.4	88.4	87.6	86.3	86.0	85.5
Acid-treated pectin solution	96.3	96.3	95.5	94.2	91.9	90.8	89.2	87.1	86.9	86.4	86.4

amount was negligible until after the first 3 hr. On the other hand, with higher temperatures such as sometimes prevail in the processing and preparation of foods, indophenol-reducing substances are formed in quantities which may seriously interfere with the determination of ascorbic acid by titration with this indicator. It is to be noted that the production of reductic acid from pectin is not accompanied by the formation of brown substances such as are produced during the action of alkalis on sugar solutions.

The results given in Table 3 show that the acid-treated pectin solution reacted with HCHO at pH 3.5 in a similar way to reductic acid, and consequently it is very probable that this reducing compound only was formed during the treatment. It may be noted here that the course of the reaction between reductic acid and HCHO was somewhat different in the buffer solution employed in the present series of experiments from that used in the experiments described in the previous paper.

Accelerating influence of some substances on the rate of disappearance of ascorbic acid in the presence of formaldehyde at pH 1.5

It was shown in our previous paper that in certain extracts the rate of disappearance of ascorbic acid in the presence of HCHO at pH 1.5 was considerably increased, thereby introducing an error into the determination. The only compound which was then found to cause an acceleration under these conditions was glycine. Further experiments have now shown that other compounds are capable of producing the same effect. Amongst these the outstanding example is methyleneaminoacetonitrile, which is far more active in this respect than glycine. Fig. 6 shows that a concentration as low as 0.5mM doubled the initial velocity of the disappearance of ascorbic acid at pH 1.5, whilst at a concentration of 40mM more than 90% of the ascorbic acid present had disappeared in 4 min. at 25°. The compound alone in the absence of HCHO did not react with ascorbic acid. It is unlikely that methyleneaminoacetonitrile occurs in any natural product, but the possibility of the occurrence of other compounds of similar activity must be borne in mind.

In our first paper we mentioned that some amino-acids did not have the accelerating effect of glycine.

Further evidence is now produced that the behaviour of other amino-acids differs from glycine quantitatively rather than qualitatively. Thus it has been found that neither glutamic acid nor alanine had a marked effect at a concentration of

Fig. 6. The rate of disappearance of ascorbic acid at pH 1.5 in the presence of HCHO and various other substances.

Curve

- A HCHO alone
- B 50mM methylamine
- C 0.5mM methyleneaminoacetonitrile
- D $\ominus$ 10mM glycine
- E $\square$ 10mM ethyl aminoacetate
- F 50mM glutamic acid
- F 50mM alanine
- G 40mM methyleneaminoacetonitrile

In cases A and C-G, HCHO added at 0 min. In case B methylamine allowed to react with HCHO for 30 min. Ascorbic acid then added at zero time. Chloride-citrate buffer. Initial concentration of ascorbic acid 0.46mM. HCHO concentration 0.52M. Temp. 25°.

10mM. When the concentration of these compounds was 50mM, however, the initial velocity of combination of ascorbic acid was greatly increased (Fig. 6). It is of interest to note that esterification of glycine did not alter its action on the rate of disappearance of ascorbic acid (Fig. 6). A very slight accelerating

effect was observed with methylamine, but only at the high concentration of 50mm, and when the HCHO was allowed to react with the amine before the addition of ascorbic acid.

Fortunately, since in the above reactions the course of the disappearance of ascorbic acid was found to be practically linear in its initial stages, it is possible to introduce a correction when acceleration is suspected at pH 1.5. This will be dealt with in a later section.

DISCUSSION

The results of this investigation leave no doubt that by heating glucose in the presence of alkali two or more indophenol-reducing substances, of which reductone may be one, are produced. The expression 'reductone' is applied here to the compound isolated by Euler & Martius (1933). This compound, purified by sublimation, was found by us to behave like the unsublimed product. The production of several indophenol-reducing substances is in accord with Euler & Klusman's (1933) suggestion that at least three 'reductones' are formed on the treatment of a hexose with alkali. Mapson also considered that two substances are formed because of the behaviour on H_2S treatment after oxidation and because the reaction with HCHO appeared to occur in two stages. The latter argument in itself, however, does not prove the dual character of the reducing substance since single pure compounds such as reductic acid or 6-substituted ascorbic acids behave in a similar manner. It is highly probable that the reducing substances formed from glucose in Euler & Martius's method of preparation consist mainly of reductone since most of the reducing value of the solution after alkali treatment could be accounted for by us in the solid compound isolated according to their instructions. Euler & Martius heated their glucose and alkali solutions in the presence of lead acetate. It is known that the action of $Pb(OH)_2$ on glucose gives rise to breakdown products different from those produced by NaOH (Löb, 1909; Löb & Pulvermacher, 1910), and as no lead salt was present in the preparation of our alkali-treated solutions it is not surprising that they exhibited properties different from reductone. In our preparations which showed progressive changes in behaviour, the properties of solution 4 (Fig. 2 and Table 2) in regard to its reaction with HCHO and reduction with H_2S after oxidation suggested the presence of reductone amongst other reducing substances.

The point which is of particular interest to us is that the reducing substances in our alkali-treated solutions behaved differently from pure reductone. The possibility of the formation of reducing substances from hexoses under conditions usually prevailing in the preparation of food is not great because

the medium is not sufficiently alkaline. When, however, higher temperatures are employed, such as are used in certain manufacturing processes, the likelihood of their formation is increased. It is of interest to note that heat and alkali, which conduce to the production of reducing substances, have at the same time a deleterious effect on ascorbic acid especially in the presence of air and consequently it would be very misleading if the resulting reducing value were taken as an index of ascorbic acid. The probability of the formation of reductic acid from pectin or pentoses, as already mentioned, might constitute a greater error in the determination of ascorbic acid in unknown solutions than the products produced from hexoses by alkali treatment. It is of importance to point out in this connexion, that whilst the production of reducing substances by the alkali treatment of glucose is accompanied by caramelization, this is not the case when reductic acid is produced. It would therefore be dangerous to assume that in the absence of caramelization no reducing substances capable of simulating the presence of ascorbic acid have been formed.

In our previous paper formulae for the correction of the results were given for solutions which contained pure reductic acid and reductone. The present investigation shows that the reductic acid formula is not applicable to unknown solutions since, if applied to reducing substances such as are found in alkali-treated glucose solutions, the error may even be increased. If, on the other hand, the reductone correction is applied, the error is definitely reduced although it may not be abolished.

In spite of the above shortcomings Lugg's formalin method still remains more specific than direct indophenol titration and if used with great caution may prove of use particularly in problems of a fundamental nature. Utilizing the knowledge so far available we have therefore worked out a procedure which to our minds yields the most satisfactory results. This procedure, which may in the first instance appear rather complicated, is really simple when put to the test.

MODIFIED PROCEDURE

Theoretical

It was our aim to simplify the method of the pH adjustments without sacrificing accuracy. In the preparation of the trichloroacetic acid extracts NaCl is added so that the pH of the acid solution is lowered and when the pH is eventually adjusted to 1.5 trichloroacetic acid is present mainly as the sodium salt. The addition of NaCl has the following advantages. The concentration of the trichloroacetic acid in the extract may under these conditions vary without greatly influencing the procedure of the pH adjustment. The high ionic concentration will eliminate any differences that may arise from the presence of inorganic constituents in the extracted material and thus avoid

changes in the velocity of the reaction which affect the accuracy of the method. Finally the NaCl will inhibit the catalytic oxidation of ascorbic acid in the presence of salts of heavy metals.

Another factor which must be considered in the adjustment of pH is the very marked influence of dilution upon pH in the 1.5 range. In the procedure described below the solution is initially adjusted to pH 1.39. This value was chosen because it has been found that, after dilution and treatment with HCHO under the conditions described, the final pH rises to exactly 1.50. At pH 3.5, on the other hand, the dilution and addition of HCHO does not alter the pH.

The adjustment of pH is carried out by addition of HCl or sodium acetate solution. Sodium acetate is used in place of alkali in order to avoid the destruction of ascorbic acid or dehydroascorbic acid by local alkalinity which might occur if NaOH were used. Over the range of the adjustment sodium acetate has a negligible buffering action and the solution is prepared so as to be equivalent in concentration to the acid used.

In order to adjust to pH 3.50 the necessary quantity of standardized sodium acetate is added to the extract which has already been brought to pH 1.39. The solution at pH 3.50 can then be adjusted to pH 1.50 by the addition of HCl equivalent to the sodium acetate added, provided that the correct conditions of dilution are observed. Thus all the data necessary for final adjustments are obtained in one operation.

As the composition of the solutions after adjustment by this procedure differs from that of the trichloroacetate-citrate buffers used in our previous investigation, we have redetermined the velocity constants of the combination of ascorbic acid with HCHO. The buffer solutions used for this purpose were also employed for all the other reaction velocity experiments in this paper, and their composition is described in the experimental section. The velocity constants are given in Table 4 together with the reaction times at different temperatures for use in the modified procedure. Since the pH adjustment involves dilution of the extracts, the volumes of the solutions have been chosen so as to simplify the calculation, and the factor for dilution is 1.2 throughout.

Table 4. *Constants for the reaction of ascorbic acid with formaldehyde at pH 1.5 and pH 3.5*

Velocity constant

$$k = (k_1 [F] + k_2) 10^3. \text{ (For symbols and units see Snow \& Zilva, 1943.)}$$

x_e = percentage ascorbic acid combined at equilibrium.

pH 1.5: the times given are those required to produce a loss of 1% ascorbic acid.

pH 3.5: the times given are those required to reach 99.5% equilibrium value.

Chloride-citrate buffers. HCHO conc.; pH 1.5, 0.52M;
pH 3.5, 0.57M.

Temp.	pH 1.5		pH 3.5		x_e
	k	Time (sec.)	k	Time (min.)	
30°	28.2	22	890	6	98.2
25°	17.5	35	554	10	98.5
20°	10.2	60	311	17	98.9
15°	5.86	105	191	28	99.1
10°	3.49	175	104	51	99.3

The reactions with HCHO are carried out in a manner similar to that recommended by Lugg. After pH adjustment, three equivalent portions of the solution are treated as follows:

- (1) Titrated at pH 1.5 with indophenol. (Titre = v' .)
- (2) Allowed to react with HCHO at pH 1.5 for the standard time (calculated to cause the combination of 1% of ascorbic acid present) and titrated. (Titre = v_1 .)
- (3) Allowed to react with HCHO at pH 3.5 for the standard time (calculated to allow the reaction with ascorbic acid to reach 99.5% of the equilibrium value), and then titrated at pH 1.5. (Titre = v_2 .)

Two different types of error may enter into the above determination, i.e. (a) due to the presence of substances which accelerate the reaction between HCHO and ascorbic acid at pH 1.5; (b) due to the presence of substances other than ascorbic acid which react with HCHO at an appreciable rate at pH 3.5. The results are therefore examined and corrections if necessary are made as follows:

- (1) When $v' - v_1 < (v_1 - v_2)/25$ and $v_2 < v_1/6$, i.e. when the errors are so small that no further corrections need be applied.

These arbitrary limits were chosen for practical purposes so that the maximum error that can arise at pH 1.5 is 3%. The limit chosen for the pH 3.5 reaction is such that in a mixture of ascorbic acid and purified reductone the maximum error in the determination of ascorbic acid would be 5%.

Then if

v = titration of ascorbic acid present in a volume of the original extract equal to that used for the titrations,

x_e = percentage ascorbic acid combined at equilibrium after HCHO reaction at pH 3.5 (Table 4),

m = titration equivalent of substances of Class 1 (Lugg's classification),

n = titration equivalent of substances of Class 3 (Lugg's classification),

$$1.2v' = v + m + n, \quad (1)$$

$$1.2v_1 = \frac{99}{100}v + n, \quad (2)$$

$$1.2v_2 = \frac{100 - x_e}{100}v + n. \quad (3)$$

Whence from (1) and (2) $v = \frac{120}{x_e - 1}(v_1 - v_2)$, the final formula used in the calculation.

- (2) When $v' - v_1 > (v_1 - v_2)/25$ and $v_2 < v_1/6$. This may be due to interference by substances of Class 1 of Lugg's classification or to substances accelerating the disappearance of ascorbic acid in the presence of HCHO at pH 1.5. A further titration is performed after reaction with HCHO at pH 1.5 for twice the standard time. (Titre = v_3 .) Then by a deduction similar to case 4 the results are calculated as follows:

$$v = \frac{120}{x_e}(2v_1 - v_2 - v_3).$$

- (3) When $v' - v_1 < (v_1 - v_2)/25$ and $v_2 > v_1/6$. This indicates the presence of a considerable quantity of substances of Class 3 of Lugg's classification which may react to some extent with HCHO at pH 3.5. A further titration is carried out after reaction with HCHO at pH 3.5 for twice the standard time. (Titre = v_4 .) Then, by a calculation similar to that given in case 4,

$$v = \frac{120}{x_e - 1}(v_1 - 2v_2 + v_4).$$

(4) When $v' - v_1 > (v_1 - v_2)/25$ and $v_2 > v_1/6$. In this case there is the possibility of interference from the sources indicated in both cases 2 and 3. Both additional titrations must then be performed and the required formula for the calculation is derived as follows:

Let r = fraction of ascorbic acid combined with HCHO at pH 1.5 in the standard time,

s = fraction of Class 3 compounds combined with HCHO at pH 3.5 in the standard time.

Assuming that the course of the reaction of ascorbic acid with HCHO at pH 1.5 in the presence of interfering substances and the reaction of Class 3 substances with HCHO at pH 3.5 are both linear, then

$$1.2v' = v + m + n, \quad (1)$$

$$1.2v_1 = v(1 - r) + n, \quad (4)$$

$$1.2v_2 = v(1 - 2r) + n, \quad (5)$$

$$1.2v_3 = \frac{100 - x_e}{100} v + n(1 - s), \quad (6)$$

$$1.2v_4 = \frac{100 - x_e}{100} v + n(1 - 2s). \quad (7)$$

Whence from equations (4), (5), (6) and (7) the final formula

$$v = \frac{120}{x_e} (2v_1 - 2v_2 - v_3 + v_4) \text{ is obtained.}$$

Practical

Solutions required. 4N-hydrochloric acid (standardized).
2N-hydrochloric acid (standardized).

Phosphate-citrate buffer made up as follows:

Citric acid	48 g.
Potassium dihydrogen phosphate	31 g.
Water	To 100 ml.

Standardized sodium acetate (approx. 4M). This solution is standardized by the following empirical procedure: 10 ml. of a solution containing approx. 57% w/v hydrated sodium acetate is titrated without dilution with 2N-HCl, with thymol blue as indicator. The end-point is taken as the first definite colour change in the acid range (yellow orange). The titration should be 20.6 ml. and the strength of the solution should be adjusted to give this value.

Preparation of extracts. Trichloroacetic acid is used for the purpose of extraction and the extracts must also contain approximately 10% w/v sodium chloride. For products containing about 80% moisture the following extracting solution is convenient, 1 ml. solution being used for each gram of tissue:

$\text{CCl}_3\text{CO}_2\text{H}$	50 g.
NaCl	180 g.
Water	To 1 l.

pH adjustment. The adjustment of pH is conveniently carried out in a glass electrode assembly having an electrode chamber of capacity approximately 50 ml. Stirring is achieved by the passage of a current of nitrogen which also prevents the oxidation of ascorbic acid in the extract by atmospheric oxygen. 4M-sodium acetate or 4N-HCl are added to the solution from 2 ml. automatic burettes. After standardization of the electrode system, 33.3 ml. extract are delivered by a special pipette into the electrode cell and 1.5 ml. phosphate-citrate buffer added. Nitrogen is bubbled through the solution and sodium acetate added slowly until the pH is exactly 1.39. If the initial pH is greater than 1.39 it may be brought to this value by the addition of HCl,

but if it is considerably higher there is an indication that the trichloroacetic acid used in the extraction was too dilute. The volume of sodium acetate or acid solution added in this preliminary adjustment need not be noted. The sodium acetate burette is then reset at zero and the extract titrated to exactly pH 3.50. If too much acetate is accidentally added the solution may be back-titrated with 4N-HCl. The volume of acetate required = u . The adjusted solution is then run into a 40 ml. graduated flask. The electrode chamber is washed out with water, and the solution and washings made up to volume and mixed. This solution can now be brought to pH 1.5 when required by the addition of $u/4$ ml. 2N-HCl/5 ml.

Three 5 ml. portions of the solution adjusted to pH 3.5 are withdrawn and treated as follows: (1) Adjusted to pH 1.5 and titrated with indophenol. (2) Adjusted to pH 1.5, allowed to react with 0.25 ml. 12M-HCHO for the time given in Table 4 according to the temperature and titrated. (3) 0.25 ml. 12M-HCHO is added and the reaction allowed to proceed at pH 3.5 for the necessary time (Table 4). The solution is then adjusted to pH 1.5 and titrated.

A further 20 ml. of the solution adjusted to pH 3.5 are treated by passing a current of H_2S for 5 min., allowed to stand for 20 hr. and the excess of H_2S removed by means of a stream of N_2 . Three 5 ml. portions of the reduced solution are then treated as above.

The results are examined and the calculation carried out as described above; further titrations are carried out if corrections are necessary.

SUMMARY

1. The production by heat of indophenol-reducing substances from glucose in the presence of alkali and from pectin in the presence of acid has been investigated under various conditions.

2. A mixture of at least two reducing substances is formed from glucose in the presence of alkali. One of these may be identical with reductone isolated by Euler & Martius (1933). The mixture of substances formed in solution behaves quite differently from reductone on treatment with HCHO or oxidation and subsequent reduction with H_2S .

3. Erroneous results are obtained when Lugg's method is applied to alkali-treated glucose solutions, and the error cannot be avoided. It is pointed out that carrying out the reaction at pH 2.0 instead of pH 3.5 is less convenient and leads to no greater accuracy.

4. Reductic acid is probably the only indophenol-reducing compound formed by the action of acid on citrous pectin. This reaction is more likely to occur under conditions similar to those used in the treatment of foodstuffs than the formation of reducing substances resembling those obtained by treating glucose with alkali.

5. The production of reductic acid occurs without the formation of deep-brown products and the danger of using caramelization as an index of the formation of non-ascorbic acid reducing substances is stressed.

6. Methyleneaminoacetonitrile is much more active than glycine in accelerating the disappearance of free ascorbic acid in the presence of HCHO at pH 1.5. The possibility of errors in Lugg's method due to other substances behaving in a similar manner is emphasized.

7. A convenient procedure based on Lugg's formalin method is described.

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The Micro-volumetric Determination of Iron, with Particular Reference to Blood

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Modern research into the biochemistry of respiratory pigments demands iron determinations of an accuracy which is beyond that of the usual colorimetric procedures. Even such sensitive and specific reagents as $\alpha\alpha'$ -dipyridyl and thiolacetic acid cannot surmount the difficulties inherent in the visual matching of colours. It is surprising, therefore, that in this field so few attempts have been made to apply to biological materials any of the volumetric methods of modern inorganic chemistry. Dubnoff & Kirk (1936) reduced iron to the ferrous state with cadmium-amalgam and titrated with standard $\text{Ce}(\text{SO}_4)_2$. They claimed to be able to determine as little as 2–15 μg . Fe, but in this laboratory all reductions with pure metals and amalgams have led to 'blanks' of several μg ., which impair greatly the accuracy of the results. Pincussen & Roman (1931) and McFarlane (1932) titrated ferric iron with standard titanous salts, but both methods require about 1 mg. Fe for each estimation. This precludes their use for many biological purposes.

This paper describes a titrimetric technique which can conveniently be used for the determination of any quantity of iron not less than 8–10 μg .

METHOD

Principle of the method. Organic matter is destroyed by heating with H_2SO_4 , HClO_4 and HNO_3

in a small pyrex flask (10–12 ml. capacity, internal neck diameter 8 mm., over-all length 80–90 mm., kindly blown for me by Mr C. Bell, Department of Biochemistry, University of Edinburgh). The digest is diluted and titrated with standard $\text{Ti}_2(\text{SO}_4)_3$, using a burette modified from the design of Conway

Fig. 1. Modified Conway burette. A, pressure-regulating vessel. B, C, D, G and H, stop-cocks. E, the burette, standard bore capillary tubing against mm. scale. 10 cm. $\approx$ 0.05 ml. F, $\text{Ti}_2(\text{SO}_4)_3$ reservoir, capacity 200–250 ml. I, digestion and titration flask.

(1939) so as to permit the storage of the $\text{Ti}_2(\text{SO}_4)_3$ under hydrogen (see Fig. 1). KCNS is used as indicator, and CO_2 for stirring.