74. The Catalytic Oxidation of Ascorbic Acid in the Presence of Tea Infusions

By G. A. SNOW and S. S. ZILVA*, *From the Division of Nutrition,
Lister Institute, London*

(Received 17 July 1942)

The experiments described in this communication originated in an observation made in connexion with a wider inquiry into the suitability of certain articles of food as carriers of ascorbic acid. It was found that condensed milk to which synthetic ascorbic acid was added, lost its antiscorbutic activity when mixed with tea. In an effort to prevent this destruction, in a way that could conveniently be applied in practice, information of theoretical interest was obtained which may have a bearing on other problems. It was established that the inactivation of the ascorbic acid was due to the presence of certain organic compounds in the infusions which were capable of catalysing the oxidation of ascorbic acid in the presence of O_2 . For the sake of convenience these catalysts will be referred to in this communication as C.P. (catalytic principles).

Preliminary observations

In this section some typical experiments are described which illustrate the destructive action of tea infusions on ascorbic acid. It was found that this deleterious effect was observed not only at 100° but also at room temperature. The early experiments were carried out in the presence of milk.

The experimental tea mixture was prepared in a series of evaporating basins each containing:

Tea	1 g.
Unsweetened proprietary condensed milk ...	4 ml.
Ascorbic acid	7 mg.
Tap water	96 ml.

The milk was diluted with part of the water and the ascorbic acid was dissolved in the diluted milk. Each solution was made up to volume and poured on the tea in the basin and mixed. The mixtures were then heated so as to bring them to the boil in about 15 min. and then kept at a temperature of about 80° for 30 min. before cooling. The loss of ascorbic acid was ascertained immediately the mixtures reached the boiling point and also at various intervals subsequently and corrections were made for diminution in volume due to evaporation. In experiments carried out at room temperature the ascorbic acid was added after the infusion had cooled. For the assessment of the ascorbic acid the mixtures were precipitated with an equal volume of 5% trichloroacetic acid. After centrifuging, an aliquot quantity from the middle layer between the fat and the coagulated protein was removed and titrated with 2:6-dichlorophenolindophenol.

It is seen from Table 1 that on reaching the boiling point about 50% of the ascorbic acid had disappeared and this disappearance continued, so that after 90 min. (30 min. at 80° and 60 min. cooling), the destruction was almost complete. A small amount of the ascorbic acid which had disappeared could be recovered as dehydroascorbic acid. At room temperature the disappearance of the ascorbic acid took place much more slowly. Even in this case the loss of the vitamin in its reduced form after 150 min. amounted to about a half. As might have been expected, more dehydroascorbic acid was found when

* Member of the Scientific Staff, Medical Research Council.

Table 1. *Loss of ascorbic acid in the presence of tea infusions containing milk*

1. Infusions brought to the boil and then maintained at approx. 80°.

		% loss of added ascorbic acid									
		Before treatment with H ₂ S					After treatment with H ₂ S				
Time min. ...		0	10	20	30	90	0	10	20	30	90
Exp. 1		33	32	46	50	—	15	16	24	38	—
2		51	69	74	81	93	46	70	64	75	92
3		59	67	—	83	88	37	56	56	69	66

(The times were measured from the point when the infusions reached 100°.)

2. Infusions kept at room temperature.

		% loss of added ascorbic acid									
		Before treatment with H ₂ S				After treatment with H ₂ S				Control, tea absent	
Time min. ...		15	30	60	150	15	30	60	150	150	
Exp. 1		19	22	32	44	—	—	—	—		3
2		13	23	31	45	0	18	—	26		3
3		9	20	33	51	5	7	10	20		2
4		11	21	34	50	0	0	2	13		4

the destruction took place at room temperature than at the higher temperatures. Only half of the destroyed ascorbic acid was irreversibly lost, the remainder being converted to the more labile, but physiologically active, dehydroascorbic acid.

Technique

In order to study this phenomenon with greater precision a more elaborate technique than the one employed in the preliminary work had to be adopted. Buffered tea infusions not containing milk were used.

The C.P. activity was determined by measuring the initial rate of oxidation of ascorbic acid at pH 7 at a temperature of 38°. The reaction was carried out in 50 ml. conical flasks supported in a thermostatically controlled water bath and shaken at the rate of 110 complete oscillations per minute which ensured the maximum rate of oxidation. The constitution of the reaction mixtures was as follows:

5 ml. aqueous solution containing the C.P. preparation and other added substances.

4 ml. phosphate buffer containing 10⁻³ M KCN.

1 ml. 0.4% solution of ascorbic acid in buffer.

The ascorbic acid solution was added after the remainder of the mixture had reached the temperature of the water bath. After a further 30 sec. the initial ascorbic acid concentration was determined in 0.5 ml. of the mixture. This was repeated six times at equal intervals ranging from 1 to 10 min. according to the activity of the C.P. The titrations were carried out in small glass thimbles of about 3 ml. capacity. The burette was so constructed that the indicator was delivered through a capillary which dipped into the solution in the thimble and which was oscillated by an electromagnetic device, thus ensuring constant and thorough stirring. Under these conditions the error did not generally exceed $\pm 0.5\%$. The most important precaution in these determinations was the control of pH, since, as will be seen later, very small deviations can cause significant changes in the rate of oxidation. Care was therefore taken to ensure that every solution in the experiments was at pH 7.0 and that the resulting reaction mixture was exactly neutral. When this was not possible the pH was determined electrometrically and corrections were made accordingly. For greater accuracy it was also desirable that in assessing the initial rate of the oxidation, measurements should be taken during the early stages of the reaction, i.e. during the oxidation of about a quarter of the total ascorbic acid present, when the rate of the reaction was only slowly diminishing.

The destruction of ascorbic acid was followed up by subtracting the subsequent titrations from the initial titrations. The obvious way of determining the initial velocity was by plotting these differences against time and drawing a smooth curve through the points. In doing this it was found that the curvature of such graphs was never great and that frequently they were almost rectilinear. The slope of the tangent at the origin was determined by the use of a tangentimeter similar to that described by Simons [1941], and from this slope the initial velocity in $\mu\text{g.}$ of ascorbic acid oxidized per min. was obtained. The form of the curves suggested that a convenient formula for calculation might be derived. Mr S. C. Pearce, of the East Malling Research Station, kindly considered the problem for us. We should like to express here our gratitude to him for the help he has given us. Mr Pearce writes: 'The relationship between the time from the beginning of the reaction, t , and the quantity of ascorbic acid present at that time, Ω_t , is approximately rectilinear, and it was decided to investigate the fit given by the simplest generalization of this relationship, namely by the second degree law

$$\Omega_t = a + bt + ct^2.$$

'Each trial yielded seven observations, $\omega_0, \omega_1, \omega_2, \dots \omega_6$, at times $0, \tau, 2\tau, \dots 6\tau$ respectively; the parameters, a, b , and c were chosen so as to make as small as possible the squared deviations between the observed and predicted results, i.e. so as to minimize $\sum_{n=0}^6 (\omega_n - \Omega_{n\tau})^2$. That this procedure is reasonable is shown by the theorem of Markoff [cf. David & Neyman, 1938], while the assumption that all observations are equally liable to error is probably justified. The curve so fitted gives the initial velocity of the reaction as

$$\frac{13\omega_0 + 2\omega_1 - 5\omega_2 - 8\omega_3 - 7\omega_4 - 2\omega_5 + 7\omega_6}{28\tau}$$

and the quantity of ascorbic acid initially present as best estimated by

$$\frac{32\omega_0 + 15\omega_1 + 3\omega_2 - 4\omega_3 - 6\omega_4 - 3\omega_5 + 5\omega_6}{42}.$$

In a series of 50 experiments the results obtained by both methods were compared and good agreement was found to exist between the two. The use of the formula was eventually adopted because it avoided subjective errors involved in drawing the curves and in estimating the slope of the tangents. The figures for reaction velocities given in the tables always refer to the loss of ascorbic acid from 10 ml. (the initial volume) of the reaction mixture. All the data given in this communication are those of representative experiments.

Catalytic nature of the oxidation

It was desirable to ascertain whether the oxidation of ascorbic acid in the presence of tea infusions was due to a non-catalytic chemical oxidation by some substance present in tea such as a quinone, or whether the reaction was due to the presence in it of substances which catalysed an oxidation process in which molecular O_2 was involved. The experiments to be described in this section leave little doubt that the latter assumption is correct.

If the substances responsible for the oxidation of ascorbic acid were acting as a catalyst no stoichiometric relationship should have existed between it and the vitamin and the oxidation should have proceeded for long periods of time. This was actually found to be the case, as will be seen from Table 2. Allowing for the loss in the control vessel, we see that 59 mg. ascorbic acid were oxidized through the agency of 20 ml. of an infusion, prepared by extracting 0.2 g. tea with 100 ml. water, which contained 15.5 mg. of dry matter, only a part of which could have consisted of the c.p. This observation is not in consonance with a non-catalytic reaction.

Table 2. *The disappearance of large quantities of ascorbic acid in the presence of tea infusion at room temperature*

Time days	mg. ascorbic acid lost		Time days	mg. ascorbic acid lost	
	Tea infusion present	Control		Tea infusion present	Control
1	14	2	4	65	16
2	31	7	5	77	18
3	49	11			

The solution contained 20 ml. tea infusion made from 0.2 g. tea extracted with 100 ml. water, 10 ml. phosphate buffer, 100 mg. ascorbic acid dissolved in 10 ml. buffer and $10^{-2} M$ KCN. In the control flask the tea infusion was replaced by water. The ascorbic acid was estimated by titration of 0.5 ml. samples at 24 hr. intervals.

Table 3. *The aerobic nature of c.p. activity*

	Contents of tubes (ml.)					
	0.5	0.5	0.5			
Ascorbic acid solution	2.0	—	—			
Tea infusion	2.5	2.5	2.5			
Buffer solution	—	2.0	—			
Benzoquinone solution	—	—	2.0			
Water						
	Air absent			Air present		
% ascorbic acid lost after 45 min.	3	93	1	88	96	18

Tea infusion was prepared from 1 g. tea extracted with 100 ml. water. Ascorbic acid solution: 20 mg. in 50 ml. water. Buffer solution: phosphate-citrate, pH 6.8. Benzoquinone solution: 0.1% in water.

In another experiment the oxidation of ascorbic acid in the presence of tea infusion or of benzoquinone was followed up in modified Thunberg tubes [Keilin, 1929] under aerobic and anaerobic conditions. In the latter case the tubes were evacuated, washed out three times with N_2 and finally evacuated, after which the ascorbic acid in the stoppers was mixed with the contents in all the tubes. Table 3 gives the details and results of the experiment. It will be noticed that whereas the vitamin was oxidized by the quinone in the absence as well as in the presence of air, in the case of the c.p., oxidation occurred only when O_2 was present. This observation proves that O_2 is utilized in the reaction. It may be mentioned in this connexion that no substance capable of effecting the anaerobic oxidation of ascorbic acid was generated in tea infusions after prolonged shaking in air.

The following experiment, which was devised mainly for the purpose of establishing the relationship between the loss of ascorbic acid through the agency of the c.p. and the absorption of O_2 , throws some light on the way the O_2 may be utilized in the reaction. The Warburg-Barcroft apparatus was employed. Each of the flasks contained a mixture of tea infusion and buffer solution in the main vessel and a solution of ascorbic acid in the side bulb. $0.5 \times 10^{-3} M$ KCN was added in every case. After the flasks had reached the temperature of the bath (38°) the ascorbic acid in the side bulbs was mixed with the solutions in the main vessels. Individual flasks were removed at intervals, the contents acidified and titrated with indophenol. At the same time the O_2 uptake was recorded in the corresponding flasks in the bath. Table 4 shows that the O_2 absorbed corresponds to the approximate uptake of 1 mol. O_2 /mol. ascorbic acid and the reaction appears to proceed thus:

That dehydroascorbic acid is formed in the reaction has already been demonstrated by us (cf. Table 1). That H_2O_2 is also produced is suggested by the results given in Table 5, which show that in the presence of catalase the O_2 uptake approximates only $\frac{1}{2}$ mol. instead of 1 mol./mol. ascorbic acid. A more accurate correspondence between the

Table 4. *Relationship between the disappearance of ascorbic acid and the absorption of O₂ in the presence of tea infusion*

Time min.	Loss of ascorbic acid μ mol.	O ₂ absorbed μ mol.	Time min.	Loss of ascorbic acid μ mol.	O ₂ absorbed μ mol.
5	—	0.29	30	1.51	1.56
10	0.68	0.65	45	1.99	2.07
15	—	0.87	60	2.61	2.52
20	1.14	1.09	75	2.96	2.90
25	—	1.32	90	3.32	3.17

Each flask contained 1.0 ml. tea infusion prepared by extracting 0.2 g. tea with 100 ml. water and 0.5 ml. phosphate buffer pH 7.0 in main vessel. 0.5 ml. ascorbic acid solution (16 mg. in 10 ml. buffer) was placed in the side bulbs and mixed with the other solutions at zero time. $0.5 \times 10^{-3} M$ HCN was present in the solutions.

Table 5. *Relationship between the disappearance of ascorbic acid and the absorption of O₂ in the presence of tea infusion and catalase*

Time min.	Loss of ascorbic acid μ mol.	O ₂ absorbed μ mol.	Time min.	Loss of ascorbic acid μ mol.	O ₂ absorbed μ mol.
5	—	0.12	45	—	1.19
10	—	0.27	50	2.61	1.31
15	—	0.50	60	3.42	1.49
20	1.59	0.55	70	—	1.65
25	—	0.73	80	3.56	1.79
30	2.00	0.87	95	3.74	2.07
35	—	0.98	120	4.20	2.37
40	2.15	1.13			

Each flask contained 0.5 ml. tea infusion prepared by extracting 0.4 g. tea with 100 ml. water, 0.5 ml. catalase preparation containing 0.16 mg. solid matter, and 0.5 ml. phosphate buffer pH 7.0 in main vessel. 0.5 ml. ascorbic acid solution (20 mg. in 10 ml. buffer) was placed in the side bulbs and mixed with the other solutions at zero time. $0.5 \times 10^{-3} M$ sodium urate was present in the solutions.

figures could not be expected because controls showed that some ascorbic acid disappeared without any uptake of O₂ in the absence of tea infusions. The latter also absorbed O₂ very slowly themselves. It may be pointed out that suitable concentrations of the catalase preparation and the C.P. had to be established by trial, since the enzyme and the C.P. in tea tended to inactivate each other.

The course of the destruction of ascorbic acid by the catalytic principles present in tea infusions

The tea infusions were buffered at pH 7.0 in $M/20$ phosphate solution. The course of the reaction in a typical experiment is shown in Fig. 1. The rate of disappearance of ascorbic acid remained fairly uniform during the first part of the reaction but gradually fell off as the concentration of the vitamin decreased. Towards the end, when most of the ascorbic acid had been oxidized, the fall in the reaction velocity became very marked.

Influence of pH on the catalytic destruction of ascorbic acid

The range chosen was from pH 5.0 to 8.0. Values lower than 5.0 tended to cause precipitation of tannin from the tea infusions, whilst above 8.0 it was impossible to avoid a rapid loss of ascorbic acid even in the absence of tea infusion. The buffer solutions used in these experiments were McIlvaine's [1921] phosphate-citrate mixtures, and the exact pH was determined by a glass electrode after addition of the tea infusion. It was found that the initial rate of reaction increased very rapidly with rise in pH. Fig. 2 shows that the log of the initial reaction velocity was directly proportional to the pH over the range investigated. This fact was later employed to provide a correction for small variations in pH in other experiments described below. From the slope of the curve in Fig. 2 it is seen that the log velocity increased by 0.65 for a rise of one pH unit.

Fig. 1.

Fig. 2.

Fig. 1. The disappearance of ascorbic acid in the presence of tea infusion. Reaction mixture: 5 ml. tea infusion prepared by extracting 0.2 g. tea with 100 ml. water, 5 ml. phosphate buffer pH 7.0, 4 mg. ascorbic acid and $10^{-3} M$ HCN.

Fig. 2. The effect of pH on the rate of oxidation of ascorbic acid in the presence of tea infusion. Infusion prepared by extracting 0.2 g. tea with 100 ml. water. Figures are calculated on the basis of 5 ml. infusion.

Fig. 3.

Fig. 4.

Fig. 3. Effect of varying tea concentrations on the rate of oxidation of ascorbic acid. Infusion prepared by extracting 0.3 g. tea with 100 ml. water.

Fig. 4. The rate of oxidation of varying concentrations of ascorbic acid by a tea infusion. Infusion prepared by extracting 0.15 g. tea with 100 ml. water. 2.5 ml. present in each flask.

Influence of tea concentration on the rate of oxidation of ascorbic acid

The rate of oxidation of ascorbic acid was measured in the presence of varying amounts of a tea infusion. A typical result is given in Fig. 3, from which it will be seen that the rate of the reaction was directly proportional to the amount of tea infusion present, a result which was in consonance with the catalytic nature of the reaction.

Influence of ascorbic acid concentration on the rate of oxidation in the presence of tea infusion

The initial rate of oxidation of ascorbic acid in the presence of a constant amount of tea infusion increased with increasing concentrations of the substrate, but not in a directly proportional manner. As will be seen from the experiment represented in Fig. 4 the influence of increasing amounts of ascorbic acid became less marked as the concentration rose, which indicated that it was possible to saturate the C.P. with excess of ascorbic acid.

The influence of various substances upon the C.P. activity of tea infusions

It was of interest to compare the catalytic action of tea infusion upon the oxidation of ascorbic acid with that brought about by Cu ions. A number of substances known to inhibit the latter type of oxidation, namely sodium chloride, cyanide, glycine, cysteine, iodide, pyrophosphate, proteins, 8-hydroxyquinoline and uric acid [Giri & Krishnamurthy, 1940], have been investigated, in order to compare their inhibiting effect on the C.P. with their action on Cu. It was necessary to correct the figures for changes in pH caused by the addition of these substances, using the method described above. The

Table 6. *Influence of various substances on the C.P. activity of tea infusions*

Added substance		pH of mixed solutions	Initial rate of oxidation $\mu\text{g.}$ ascorbic acid per min.		
Name	Concentration		Observed	Corrected to pH 7.00	% of rate in absence of added substances
None	—	7.00	38.8	38.8	—
Sodium chloride	0.1 M	6.84	40.0	50.8	131
Potassium iodide	0.1 M	6.90	59.4	69.0	178
Sodium pyrophosphate	0.01 M	6.98	36.6	37.8	97
Glycine	0.05 M	6.97	30.8	32.2	83
8-Hydroxyquinoline	0.005 M	7.12	41.4	34.6	89
Dimedone	0.1 %	6.90	24.0	27.8	72
Egg albumin	0.2 %	7.03	19.0	18.2	47
Cysteine	0.002 M	7.00	24.4	24.4	63

Each flask contained 2.5 ml. tea infusion prepared by extracting 0.2 g. tea with 100 ml. water, 2.5 ml. of an aqueous solution of the added substance adjusted to pH 7.0, 5 ml. phosphate buffer pH 7.0, 4 mg. ascorbic acid and 0.5×10^{-3} M HCN.

Table 7. *Influence of cyanide and urate on the C.P. activity of tea infusions*

Added substance	Concentration $M \times 10^{-4}$	Initial rate of reaction, $\mu\text{g.}$ ascorbic acid per min.	% inhibition due to added substance
None	—	45.2	—
Potassium cyanide	1	30.6	32
"	3	26.8	41
"	10	21.6	52
"	30	14.4	68
"	100	10.4	77
Sodium urate	5	38.4	15

Each flask contained 2.5 ml. tea infusion prepared by extracting 0.15 g. tea with 100 ml. water, 2.5 ml. of an aqueous solution of the added substance adjusted to pH 7.0, 5 ml. phosphate buffer pH 7.0 and 4 mg. ascorbic acid.

results are shown in Tables 6 and 7. Sodium pyrophosphate, glycine and 8-hydroxyquinoline (Table 6) and sodium urate (Table 7) caused little alteration in the velocity of oxidation, whilst egg albumin and cysteine somewhat inhibited it. The inhibitory action of the protein may be explained by its precipitating effect on the tannins. Milk also exercised a protective action which was probably due to the presence in it of protein. The retarding action of cysteine can be explained by its reducing action on the dehydroascorbic acid formed in the reaction [cf. Borsook, Davenport, Jeffreys & Warner, 1937]. Dimedone which reacts with quinones had also some retarding action. The accelerating effect of NaCl and KI cannot at present be explained.

Fig. 5. Inhibition of the c.p. activity of a tea infusion by varying concentrations of boric acid. Infusion prepared by extracting 0.4 g. tea with 100 ml. water. 2.5 ml. present in each flask. The points given represent experimental results. The graph drawn is the hyperbola $y = \frac{100x}{2.47 + x}$.

The inhibitory action of cyanide on the Cu-catalysed oxidation of ascorbic acid is very powerful and this reagent was therefore specially studied at various concentrations. Table 7 shows that a cyanide concentration as low as $10^{-4}M$ caused a reduction in the reaction velocity, but that increasing amounts of cyanide did not lead to a proportionate degree of inhibition. Thus, low concentrations of cyanide did not mask the action of the c.p. but probably suppressed other catalytic effects. In view of this observation it was thought advisable to carry out all the experiments in the presence of $0.5 \times 10^{-3}M$ cyanide, in order to ensure a higher degree of specificity. Boric acid was found to be a powerful and apparently specific inhibitor of the reaction under investigation. Fig. 5 shows the extent of inhibition produced by varying concentrations of this reagent. It will be seen that, except for the two highest concentrations, the points lie along a hyper-

Table 8. *The inhibiting action of boric acid on the activity of tea infusions of varying concentrations*

Vol. of tea infusion ml.	Initial rate of oxidation, $\mu g.$ ascorbic acid per min.		% inhibition due to boric acid
	Boric acid absent	Boric acid present	
2.5	103	79.0	23
1.25	51.5*	32.8	36
0.8	33.0*	17.8	46
0.5	20.6*	10.0	51

* Calculated from figure for 2.5 ml. tea infusions.

The tea infusion was prepared by extracting 0.4 g. tea with 100 ml. water. Each flask contained the volume of infusion stated above, together with 5 ml. phosphate buffer pH 7.0, 4 mg. ascorbic acid, $0.5 \times 10^{-3}M$ HCN and water to bring the total volume to 10 ml. When boric acid was present its concentration was $0.5 \times 10^{-4}M$.

bola. It would therefore appear that boric acid formed with the C.P., or with the ascorbic acid, dissociating complexes which had little or no activity. That the complexes were formed with the C.P. is suggested by the results given in Table 8, from which it is seen that the inhibition produced by a given concentration of boric acid became greater as the strength of the tea infusion was diminished. The reaction rates for different concentrations of tea infusion in the absence of boric acid were here calculated from figures obtained at one concentration, by means of the rectilinear relationship which has already been shown to exist between the velocity of oxidation and tea infusion concentration (cf. Fig. 3).

Solubility

The C.P. were found to be insoluble in ethyl acetate, alcohol, acetone, chloroform and benzene. Prolonged extraction with ether removed a considerable amount of matter but comparatively little of the C.P.; the bulk of the active substance, about eight times that present in the ether extract, remained behind. Acetone and alcohol containing 15–50 % of water were found to be effective in removing the C.P., but unfortunately these solvents removed at the same time some highly coloured substances from the tea and were therefore less suitable than water in which the C.P. were only sparingly soluble. The conditions for extraction with water were therefore studied in detail. A series of infusions was made with distilled water using varying amounts of tea. The tea and water were mixed, brought to the boil, and boiled vigorously for 10 min. with continual stirring. The leaves were then removed by straining through muslin and squeezed tightly to remove as much liquid as possible. The infusions were cooled, brought to pH 7.0 and allowed to stand overnight. All infusions more than 0.6 % in strength were found to be turbid and showed a tendency to precipitate on standing. This turbidity disappeared however on the addition of the phosphate buffer in the determination of the C.P. It would appear from Table 9 that the efficiency of the extraction diminished with the increase in the strength of the tea infusions, particularly in those from more than 0.3 g. tea per 100 ml. solution. This point emerges particularly clearly from the ratio of the C.P. activity to the weight of dry matter in the various extracts.

In spite of the sparing solubility of the C.P. in water we found that fairly active solutions could be obtained without heat by extracting ground tea with cold water for several hours with constant stirring.

Table 9. *The efficiency of aqueous extraction of tea at various concentration levels*

Strength of infusion, g. tea extracted with 100 ml. water	Vol. of infusion taken ml.	Initial rate of oxidation $\mu\text{g. ascorbic acid per min.}$	Wt. of residue from 100 ml. infusion g.	% extraction*		Initial rate of oxidation, $\mu\text{g. ascorbic acid per min. per mg. solid matter}$
				C.P. activity	Total solids	
0.1	5	55.4	0.0387	—	—	28.6
0.2	5	112	0.0780	101	101	28.7
0.3	3.5	108	0.115	93	99	26.8
0.6	1.8	92.6	0.223	77	96	23.0
1	1.0	54.2	0.366	49	95	14.8
2	1.0	96.8	0.708	44	91	13.7
4	0.8	116	1.22	33	79	11.9
7	0.6	85.6	1.83	18	68	7.8
10	0.5	108	2.48	19	64	8.7

* Figures based on assumption of 100 % extraction for 0.1 % infusion.

The action of alkali on the C.P. activity of tea infusions

Two tea infusions were used, one prepared from untreated tea and one from tea which had previously been exhaustively extracted with ether; both infusions were adjusted to pH 7.0 before use. The effect of alkali treatment under varying conditions is shown in Table 10.

Table 10. *The action of alkali on the C.P. activity of tea infusions*

Treatment	Infusion A			Infusion B		
	N NaOH absorbed per 100 ml. infusion ml.	Initial rate of oxidation μ g. ascorbic acid per min.	% of reaction rate compared with untreated infusion	N NaOH absorbed per 100 ml. infusion ml.	Initial rate of oxidation μ g. ascorbic acid per min.	% of reaction rate compared with untreated infusion
None	—	162	—	—	89.2	—
Alkaline solution kept 1 hr. at room temperature	0.07	154	95	0.06	80.3	90
Alkaline solution boiled 1 hr. under reflux in an atmosphere of N ₂	0.26	349	215	0.18	137	154
Alkaline solution kept 24 hr. at room temperature in air	0.87	65.6	40	0.31	34.6	39
Alkaline solution kept 24 hr. at room temperature <i>in vacuo</i>	0.11	186	115	0.08	127	143
Alkaline solution aerated for 6 hr.	0.62	59.2	37	0.75	55.8	63
Neutral solution aerated for 6 hr.	—	185	114	—	94.6	106

Infusion A = 0.4 g. tea extracted with 100 ml. water. Infusion B = 0.4 g. ether-extracted tea extracted with 100 ml. water. In solutions treated with alkali 2 ml. N NaOH were added per 100 ml. infusion. The results are calculated on the basis of 5 ml. of the undiluted infusions.

When the infusions were treated with one-fiftieth of their volume of N NaOH, allowed to stand for 1 hr. at room temperature and then brought to pH 7.0 by addition of HCl, little change in the C.P. activity was noted. When however the alkaline infusions were maintained at 100° in an atmosphere of N₂ for the same length of time, a marked increase in the C.P. activity resulted and a small amount of the added alkali disappeared. It should be noted that this treatment with NaOH caused a greater increase in C.P. activity with the normal tea infusion than with that prepared from the ether-extracted tea.

The effect of more prolonged exposure of the infusions to alkali at room temperature was also studied. It will be seen that after 24 hr. of the alkali treatment in air there was no increase in activity; on the contrary this treatment led to a marked decrease in activity, at the same time some absorption of NaOH also occurred. When however air was excluded a slight increase in activity was observed, the disappearance of the alkali being only slight. The aeration of an alkaline solution for a shorter period had also an inactivating effect, whilst the passage of air through the infusions at pH 7.0 caused little alteration in activity. It would appear that the C.P. are inactivated by oxidation and that this destruction proceeds at an appreciable rate in alkaline solutions exposed to air.

Acetylation and benzoylation of tea preparations

These experiments were carried out in an attempt to isolate the active principles. Dry preparations exhibiting C.P. activity were used in preparing the derivatives. In order to obtain fuller information two different methods of extraction were employed.

Preparation 1. 10 g. tea were boiled for 10 min. with 5 l. water and then filtered and concentrated to 100 ml. at atmospheric pressure. The active solid matter which separated was filtered off, dried, washed with acetone and finally dried *in vacuo*. The yield was 1.15 g., representing approximately 30 % of the solid matter extracted.

Preparation 2. 250 g. tea were ground finely, mixed with 1 l. cold water and heated to about 100°. The tea was then thoroughly pressed to remove the absorbed water and dried.

150 g. of the dried product, after extraction with ether in a Soxhlet apparatus for 240 hr. (residue 130 g.), were boiled for 1 hr. with a mixture of 5 l. acetone and 1.5 l. water. After filtration the extract was evaporated to dryness under reduced pressure; it yielded 12.5 g. solid active material.

Acetylation of the solid preparations was accomplished by refluxing 1 g. of the material with 1 g. of fused sodium acetate and 5 ml. of acetic anhydride for 1 hr. The products were separated by pouring the mixture into water and were thoroughly washed and dried. Benzoylation was carried out by the usual Schotten-Baumann method. 1 g. of the tea preparation was dissolved in 10 ml. 2*N* NaOH and treated with 4 ml. benzoyl chloride. The product which separated out was thoroughly washed, dried, dissolved in chloroform and precipitated with light petroleum.

It soon became evident that by these esterifications at least two fractions could be obtained from each derivative, one of which was soluble in benzene or ether and the other insoluble. Both fractions showed activity on hydrolysis. The relative amounts of the two fractions varied according to the method used in preparing the tea extract. None of the acetylated or benzoylated products was appreciably soluble in water or in cold NaOH. Suspensions obtained by diluting acetone solutions with water were found to be entirely devoid of C.P. activity. It was not possible to recrystallize any of the derivatives. Although they were fairly soluble in acetone, chloroform or acetic acid, concentration of the solutions led merely to the precipitation of amorphous material. The above derivatives were not active in themselves but became so on hydrolysis with dilute aqueous NaOH. The fractions insoluble in benzene were fully hydrolysed by 0.1*N* alkali at 100° in 10–15 min., whilst the benzene-soluble fractions required a somewhat longer time with 0.5*N* NaOH. The hydrolysed products were examined for C.P. activity by the usual method. In the case of the acetyl derivatives the acetyl values were at the same time determined by steam distillation. The activity of the preparations was calculated per mg. of the dry product before hydrolysis. In this way it was possible to calculate the degree of recovery of the C.P. in the hydrolysed products by comparison with the original tea preparation treated with NaOH under similar conditions. The figures given in Table 11

Table 11. *Acetyl and benzoyl derivatives of active tea preparations*

Derivative		% benzene-soluble fraction in crude derivative	Acetyl content % CH_3CO	C.P. activity of solutions obtained by alkaline hydrolysis	
				Initial rate of oxidation $\mu\text{g. ascorbic acid per min. per mg. derivative}$	% recovery compared with activity of original preparation
<i>Tea preparation 1</i>		—	—	32.6	100
Acetyl derivative from 1	Benzene-soluble fraction	19	33.4	16.6	76
	Benzene-insoluble fraction	—	34.5	23.8	111
Benzoyl derivative from 1	Benzene-soluble fraction	24	—	6.2	42*
	Benzene-insoluble fraction	—	—	10.8	76*
<i>Tea preparation 2</i>		—	—	51.6	100
Acetyl derivative from 2	Benzene-soluble fraction	52	38.7	32.0	101
	Benzene-insoluble fraction	—	37.2	35.0	108

* Calculated on the assumption that benzoyl derivatives have a similar constitution to that of the corresponding acetyl products.

indicate that the benzene-soluble and benzene-insoluble fractions differed little in their acetyl contents; in general both showed an activity of a similar order to that of the original preparation after allowance had been made for the weight of acetyl present. As benzoyl values could not be conveniently obtained, calculations were made for these

derivatives assuming that they had the same constitution as the acetyl products. On this assumption it would appear that the recovery of the C.P. after benzylation is not so great as after acetylation.

Attempted isolation of the catalytic principles

The activity was entirely removed by precipitation with an excess of neutral Pb acetate and could be regenerated from the precipitate by treatment with H_2SO_4 in an aqueous acetone solution. Attempts to concentrate the active material by fractional precipitation by successive additions of Pb acetate or by variation of the pH were unsuccessful. A partial removal of the C.P. activity by precipitation could also be brought about by saturating the infusions with NaCl, or by the addition of mineral acid or caffeine. The catalytic material was readily absorbed by aluminium hydroxide precipitated in the solution, and less easily by 'active' Al_2O_3 or norit charcoal. Some elution could be effected from the latter absorbent by means of aqueous acetone, but in the case of Al_2O_3 the combination appeared to be chemical rather than physical and it was therefore not found possible to elute the active substance. Equally unsuccessful were attempts at the separation from buffered tea infusions by the chromatographic method using the following absorbents: calcium carbonate, calcium sulphate, kieselguhr and silica. The preparation of *p*-azobenzenebenzoic acid esters and their subsequent separation by chromatography also did not yield satisfactory results in our hands.

As has already been mentioned, the C.P. activity is lost on benzylation or acetylation, is recovered after hydrolysis of the esters and is slowly lost by aerobic oxidation in alkaline solution. All these properties correspond with those of the polymerized tannins present in manufactured tea and it was therefore reasonable to suppose that the substances responsible for catalysing the oxidation of ascorbic acid were closely associated with the tannin fraction. The inhibitory action of boric acid also suggested that the active substances probably contained several hydroxyl groups. As preliminary attempts had shown that the isolation of the active substances from tea infusions was associated with great difficulties, it was thought worth while to investigate the activity of substances which, in common with the tea tannins, possess several phenolic hydroxyl groups. The following compounds were therefore tested for their catalytic action on the oxidation of ascorbic acid in the presence of cyanide: *d*-catechin, a stereoisomeride of *l*-epicatechin, which is an important constituent of the tannin fraction in fresh tea leaves [Tsujimura, 1929; Deijs, 1939], tannic acid, catechol, quinol, pyrogallol and gallic acid (Table 12).

Table 12. *Oxidation of ascorbic acid in the presence of various polyhydric phenols*

Compound		Initial rate of oxidation of ascorbic acid $\mu\text{g. per min.}$	Initial rate of oxidation of ascorbic acid $\mu\text{g. per min. per}$ mg. polyhydric phenol
Name	mg. per ml. reaction mixture		
Catechol	5	<2	<0.04
Quinol	5	48.6	0.97
Pyrogallol	5	24.8	0.50
Gallic acid (commercial sample)	2.5	163	6.5
Gallic acid (four times recryst. from water)	1.5	243	16.2
Tannic acid (b.p.)	5	22.6	0.45
<i>d</i> -Catechin	5	<2	<0.04

d-Catechin was prepared from cube gambier [Freudenberg & Purrmann, 1924]. The tannic acid was a commercial sample presumably consisting mainly of tannins derived from gallic acid; the other reagents were also commercial specimens. Considering that the above compounds were not of the highest purity the very low activity observed may not

have been due to the compounds themselves. Only gallic acid showed any appreciable activity, and even in this case less soluble impurities which became concentrated on repeated crystallization were probably responsible for the catalytic action.

DISCUSSION

There can be little doubt that the aerobic oxidation of ascorbic acid in the presence of tea infusions is catalysed by non-metallic constituents. The oxidation involves the absorption of 1 mol. O_2 /mol. ascorbic acid and leads to the production of dehydroascorbic acid and H_2O_2 . This could occur according to either of the following two schemes:

The latter scheme postulates a very low concentration of the C.P. in the tea infusions, since no appreciable oxidation of ascorbic acid occurs in the absence of O_2 .

In this investigation all the experiments were carried out in the presence of cyanide, thus excluding the action of metallic, and possibly other, catalysts. The evidence however points to the fact that more than one organic substance capable of catalysing the oxidation was involved in our experiments. It was seen that by extracting the benzoyl and acetyl derivatives with benzene, two fractions were obtained, both of which showed activity on hydrolysis, and also that exhaustive extraction of dry tea leaves with ether removed an active fraction leaving a residue with considerable activity. Although we have not succeeded yet in isolating the active substances the indications are that they are closely associated with, or chemically related to, the tannin fraction of the tea extracts.

The possibility cannot be excluded that substances of a similar catalytic nature may be present in other vegetable and fruit tissues, either originally or after treatments such as cooking, drying etc. In fact, without going into the matter exhaustively, we have already observed that other vegetables exhibit this catalytic activity. Thus aqueous acetone extracts of the root and stem of rhubarb and aqueous infusions of the dried leaves of rhubarb and camelia were found to be active. These may be exceptional sources, but should the presence or formation of such catalytic substances in vegetables and fruits be fairly common it would add one more factor to those which have to be considered in connexion with the stability of vitamin C in vegetables. The problem therefore calls for further inquiry.

SUMMARY

Tea infusions contain constituents capable of catalysing the aerobic oxidation of ascorbic acid in the presence of cyanide. Evidence is produced which suggests that these constituents are closely associated with or chemically related to the tannin fraction of the tea extracts.

A commercial sample of gallic acid showed some catalytic activity whilst *d*-catechin, tannic acid, catechol, quinol and pyrogallol were almost inactive.

The rate of the catalytic reaction has been studied in the presence of varying concentrations of tea and of ascorbic acid and at different *pH* values; it considerably decreases (*ca.* 95 %) between *pH* 7 and 5.

Milk, egg albumin, cysteine and, to some extent, dimedone inhibited the catalytic action, whilst NaCl and KI accelerated it. Sodium pyrophosphate, glycine, 8-hydroxy-quinoline and sodium urate did not appreciably influence the velocity of oxidation.

Benzoyl and acetyl derivatives obtained from the extracts, although not themselves active, became so on hydrolysis with alkali at 100°. The alkaline treatment of tea infusions at 100° under anaerobic conditions increased their catalytic activity.

The active constituents of tea infusions were slowly decomposed in alkaline medium by air.

The active constituents formed inactive complexes with boric acid.

The possible mechanisms involved in this catalytic oxidation of ascorbic acid are discussed.

It is pointed out that the presence or formation of catalytic substances of this nature in vegetables and fruits may have to be considered as an additional factor in the stability of vitamin C in vegetables and fruits.

We should like to take this opportunity of expressing our gratitude to Dr R. G. Hatton, Director of East Malling Research Station, for laboratory accommodation. Thanks are also due to Messrs Roche Products Ltd. for a gift of ascorbic acid. One of us (G. A. S.) is indebted to the Medical Research Council for a whole-time grant.

REFERENCES

- Borsook, H., Davenport, H. W., Jeffreys, C. E. P. & Warner, R. C. [1937]. *J. biol. Chem.* **117**, 237.
David, F. N. & Neyman, J. [1938]. *Statist. res. Mem.* **2**, 105.
Deijs, W. B. [1939]. *Rec. Trav. chim. Pays-Bas*, **58**, 805.
Freudenberg, K. & Purrman, L. [1924]. *Liebigs Ann.* **437**, 274.
Giri, K. V. & Krishnamurthy, P. V. [1940]. *Nature, Lond.*, **147**, 59.
Keilin, D. [1929]. *Proc. roy. Soc. B*, **104**, 213.
McIlvaine, T. C. [1921]. *J. biol. Chem.* **49**, 183.
Simons, H. P. [1941]. *Industr. Engng Chem.* (Anal. ed.), **13**, 563.
Tsuji-mura, M. [1929]. *Sci. Pap. Inst. phys. chem. Res. Tokyo*, **10**, 252.