CVI. THE ANTISCORBUTIC FRACTION OF LEMON JUICE. X.

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Owing to the difficulty of isolating vitamin C attempts were made in the present series of investigations to obtain some evidence of the chemical nature of this vitamin indirectly by studying the mode of its inactivation under various conditions. A fact observed some time ago in this connection was that when decitrated lemon juice was autoclaved for 1 hour under anaerobic conditions [Zilva, 1927] the reducing principle and vitamin C in the juice disappeared more rapidly than they did in un-autoclaved juices. Further experiments [Zilva, 1929] produced suggestive evidence that a substance, possibly of a phenolic character, was formed in the process of autoclaving which conduced to the increased rate of destruction of the reducing principle and the antiscorbutic factor.

In the experiments to be described we made an endeavour to throw further light upon this phenomenon.

We have now been able to show that when the reducing sugars (invert sugar [Zilva, 1924]) were removed from the juice by anaerobic fermentation, the spontaneous inactivation of the reducing principle or of vitamin C was no longer accelerated by the process of autoclaving. Further, when solutions of glucose, fructose or invert sugar containing calcium citrate, Rochelle salt, sodium carbonate or dipotassium hydrogen phosphate were autoclaved under the same conditions as the decitrated lemon juice they acquired the property of accelerating the destruction of the reducing principle and vitamin C. This to our mind proves conclusively that the factor formed in the process of autoclaving which increases the rate of inactivation of the vitamin complex is formed from the sugar present in the lemon juice.

Attempts to isolate and identify this substance did not meet with success owing to the very small quantities formed. Nevertheless from a comparison of the chemical properties of the ethereal extracts of autoclaved sugar solutions and autoclaved decitrated lemon juice with those of the di- and trihydric phenols, satisfactory evidence was obtained that a substance or

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substances resembling catechol or some of its derivatives was formed as the result of autoclaving.

In the course of this work it was also observed that dihydric phenols were unable to accelerate the destruction of the reducing principle in the absence of an enzymic factor though their oxidation products such as quinone *etc.* could do so unaided. The "thermostable peroxidase" [Zilva, 1929] does not seem to take any part in this mechanism of inactivation.

The preparation and properties of the ethereal extract of autoclaved decitrated lemon juice.

Method of extraction. Decitrated lemon juice adjusted to p_H 7 was autoclaved under anaerobic conditions for 1 hour at 40 lbs. pressure as described in a previous communication. After cooling and filtering, the reaction was again adjusted to p_H 7 and the juice shaken for 30 mins. in a mechanical shaker with $1\frac{1}{4}$–$1\frac{1}{2}$ volumes of peroxide-free ether. Batches of 200 cc. of juice were extracted in stoppered bottles of 600 cc. capacity, the air being displaced by CO_2 prior to shaking. The ethereal layer after separation was first concentrated at 35° under reduced pressure to a small volume and was finally dried in an evacuated desiccator over sulphuric acid. A yield of about 0·2 g. of a black-brown, tarry residue was thus obtained per litre of juice. A second extraction of the juice did not remove any appreciable further quantity nor was there any improvement in the yield when the juice was extracted by a continuous method for periods ranging from 3 to 5 hours.

Solubility. The residue, after being dried, redissolved in water or ether only with difficulty, the aqueous solution being neutral to litmus. It was soluble in ethyl or methyl alcohol, acetone and glacial acetic acid. It dissolved in dilute alkali carbonate and alkalis giving a brown solution and in sulphuric acid with a red colour. It was not reprecipitated from alcoholic solution by ether but the addition of water caused a turbidity.

Chemical properties. It contained only traces of nitrogen, but no phosphorus, sulphur or halogens. It decolorised both acid and alkaline $KMnO_4$ immediately in the cold and absorbed bromine and iodine from aqueous solutions. It reduced ammoniacal $AgNO_3$ instantly, but Fehling's solution only slowly in the cold and gave a Prussian blue precipitate with a mixture of $FeCl_3$ and K_3FeCy_6. A precipitate was formed in the aqueous solution on the addition of bromine. With $FeCl_3$ it gave a brown coloration which turned red upon the addition of a weak alkali, a reaction resembling that of catechol compounds. Tests for peroxide, with starch and KI, $K_2Cr_2O_7$ and titanium sulphate, were negative even after solutions of the residue had been kept in air for several days.

The properties of autoclaved sugar solutions and the preparation and properties of their ethereal extracts.

In attempting to trace the constituent of decitrated lemon juice from which this ether-soluble substance was formed, attention was focused upon the sugar content of the juice, because of the work of Hoppe-Seyler [1870], Bernhauer and Nepp [1931] and others who have shown that phenolic substances are formed on heating sugars and carbohydrates at high temperatures in the presence of alkalis and also because of the following observations made by ourselves. We found that when the reducing sugar in decitrated lemon juice was fermented the iodine-absorbing capacity of the juice did not increase on autoclaving and, further, the addition of such autoclaved juice to ordinary decitrated lemon juice did not accelerate the destruction of the reducing principle as does that of an autoclaved unfermented juice. This is shown by the following experiment. 200 cc. of decitrated lemon juice at p_H 7·0 together with 5 g. of calcium carbonate were placed in an ampoule which was then exhausted and kept at 38°–40° for 24 hours. After this time practically the whole of the invert sugar had been spontaneously fermented. The solution was then filtered and the filtrate autoclaved under strictly anaerobic conditions for 1 hour at 143° [see Zilva, 1928]. 100 cc. of the autoclaved solution after cooling and filtering were evaporated to dryness under reduced pressure at 40° and the residue dissolved in 100 cc. of untreated decitrated lemon juice. A batch of decitrated lemon juice which had not been previously fermented was treated similarly. These two solutions as well as a control batch of the same decitrated lemon juice were stored aerobically in a cold room and titrated periodically with 0·02 % phenolindophenol solution. As will be seen from Fig. 1, the destruction of the reducing principle in the control and in the solution containing the residue from the autoclaved, fermented, decitrated lemon juice proceeded at the same rate, whilst in the decitrated juice containing the residue from the autoclaved, unfermented, juice, the disappearance of the principle was greatly accelerated.

The effect of autoclaving sugar solutions was therefore next studied. It was found that autoclaving 1 % solutions of glucose, fructose or invert sugar, (decitrated lemon juice usually contains about 1·0 % of invert sugar), did not cause them to absorb iodine in neutral solution, nor did the addition of their residues (obtained by evaporation under reduced pressure at 40°) to neutral decitrated lemon juice accelerate the destruction of the reducing principle in the juice on storage in air. This will be seen from Fig. 2. On the other hand, when 1 % solutions of these sugars containing 0·25 % of sodium carbonate, dipotassium phosphate, Rochelle salt or sodium citrate were autoclaved anaerobically for 1 hour at 143° they exhibited the same property of accelerating the inactivation of the reducing principle in neutral solution as autoclaved decitrated lemon juice (Fig. 2). The same result was obtained with an autoclaved 1 % invert sugar solution containing 8 % citric acid (lemon juice usually

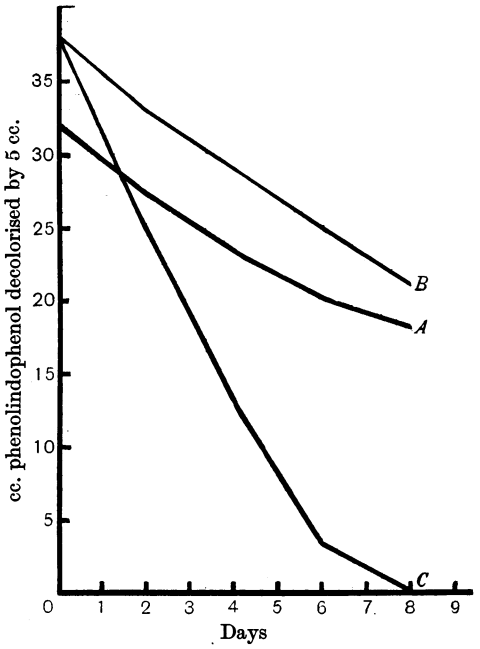

Fig. 1. *A*, decitrated lemon juice; *B*, decitrated lemon juice containing residue from autoclaved fermented decitrated lemon juice; *C*, decitrated lemon juice containing residue from autoclaved decitrated lemon juice.

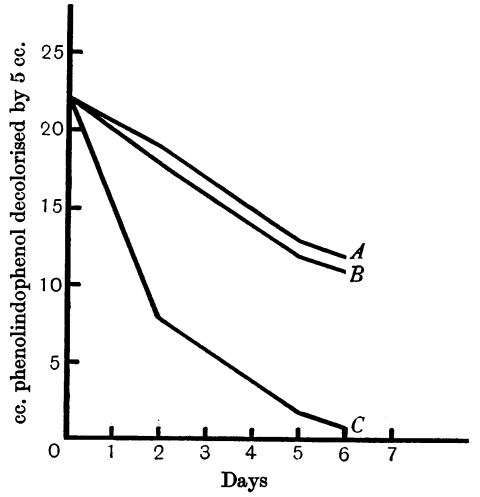

Fig. 2. *A*, decitrated lemon juice; *B*, decitrated lemon juice containing residue from autoclaved 1 % invert sugar solution; *C*, decitrated lemon juice containing residue from an autoclaved solution of 1 % invert sugar–0·25 % sodium carbonate mixture.

contains about this amount of citric acid) which had been decitrated with calcium carbonate. An ethereal extract prepared from a 1·0 % invert sugar solution containing 0·25 % of sodium carbonate, which had been autoclaved, showed identical chemical properties with the ethereal extract obtained from autoclaved lemon juice described above. In this case the ferric chloride reaction for catechol compounds was unmistakable.

The addition of the residue from an autoclaved solution of a mixture of sugar and sodium carbonate to decitrated lemon juice also increased the rate of destruction of vitamin C. This is shown by the following biological experiment.

The residue from 100 cc. of an autoclaved solution of a mixture of 1 % invert sugar and 0·25 % of sodium carbonate, obtained by evaporation under reduced pressure at 40–45°, was dissolved in 100 cc. of decitrated lemon juice. This solution, as well as a control batch of the same decitrated lemon juice stored at neutral reaction for 5 days, was tested for its antiscorbutic potency. Four guinea-pigs on a daily dose of 3 cc. and 3 guinea-pigs on a daily dose of 5 cc. of the control juice showed no, or very mild, signs of scurvy at the autopsy when chloroformed after 60 days. In contrast to this 7 guinea-pigs receiving daily 3 cc. of the stored decitrated lemon juice containing the residue from an autoclaved solution of sugar-carbonate mixture and 5 guinea-pigs on a daily dose of 5 cc. of the same preparation died of the disease within 45–60 days. The addition of this residue to decitrated lemon juice is thus seen to produce the same effect upon the vitamin C of the juice as that produced by the addition of autoclaved decitrated lemon juice [Zilva, 1928, 1929].

A comparison of the chemical properties of the ethereal extracts with those of polyhydric phenols.

Various attempts have shown that the isolation from autoclaved decitrated lemon juice of the substance which accelerates the destruction of the reducing principle and of vitamin C is a task fraught with many difficulties. However, in view of the phenolic properties of the ethereal extracts of autoclaved decitrated lemon juice and sugar solutions, it was considered that a comparison of the chemical properties of these extracts with those of polyhydric phenols might yield some indirect information on the subject.

In addition to their ability to react with iodine in neutral solutions the ethereal extracts and the polyhydric phenols also react with iodine in alkaline solutions, iodoform separating during the reaction in each case. The quantitative nature of these reactions with iodine was therefore studied in detail.

The following phenols were investigated, catechol, guaiacol, homocatechol, protocatechuic acid, resorcinol, quinol, phloroglucinol, pyrogallol and hydroxyquinol.

The trihydric phenols and resorcinol and guaiacol were found as a result of preliminary experiments to be quite dissimilar in their chemical reactions from the ethereal extracts and were therefore not investigated further.

The reactions of the ethereal extracts with iodine. One litre of decitrated lemon juice at p_H 7·0 was autoclaved in the usual manner and after cooling and filtering was acidified with 7 cc. of 4*N* HCl. The juice was then extracted by shaking with 1200 cc. of B.P. ether for 30 min. as described earlier. The ethereal layer was separated, dried over $CaCl_2$, filtered and then evaporated to dryness under reduced pressure. The residue was dissolved as completely as possible with three lots of 7 cc. of methyl alcohol and finally made up at p_H 7·0 with water to 100 cc. An extract was prepared in the same manner from 1 litre of a solution containing 1 % invert sugar and 0·25 % Na_2CO_3 which had been autoclaved under the same conditions as the lemon juice. The solution of the extract from the autoclaved decitrated lemon juice was very turbid, whilst that from the autoclaved sugar solution was only slightly so. The material causing the turbidity could not be filtered off nor did it settle out as a sediment after standing several days.

Five cc. of each of these solutions were mixed with 10 cc. of *N*/100 I and, after standing 5 minutes, the unabsorbed excess was determined with *N*/100 sodium thiosulphate. The extract from the juice absorbed 3·6 cc. *N*/100 I and that from the autoclaved sugar solutions 3·8 cc. *N*/100 I. With these extracts a very rapid absorption of iodine occurred in 5 mins., further absorption took place very slowly, as will be seen from Table I. The reactions of iodine with the phenols, to be referred to later, were complete in 5 mins., when a suitable quantity of iodine was employed. We therefore assumed that the iodine absorption which took place during the first 5 mins. in the case of these extracts was comparable to that of the phenols.

Table I.

	cc. of *N*/100 iodine used up by 5 cc. at neutral reaction in		
Solution of ethereal extract from	5 min.	1 hr.	24 hrs.
Autoclaved decitrated lemon juice	3·6	4·4	6·2
Autoclaved solution of 1·0 % invert sugar and 0·25 % sodium carbonate mixture	3·8	4·7	7·6

Five cc. of each of these extracts were next mixed with 10 cc. *N*/10 I after which 1 cc. of *N* NaOH was added drop by drop with gentle shaking. The solutions after standing 10 min. were acidified with 1 cc. of 4*N* H_2SO_4 and the excess iodine determined with *N*/10 sodium thiosulphate. The extract from the autoclaved juice used up 2·8 cc. *N*/10 I whilst the other extract used 3·0 cc. *N*/10 I. Very small amounts of iodoform separated during both these reactions, which were found to be completed in the time employed.

The ratios between the amounts of iodine used up in the reactions in neutral solution and the amounts used up in the reactions in alkaline solution, *viz.* 1:7·8 in the case of the extract from the autoclaved juice and 1:7·9 in the case of the extract from the autoclaved sugar solution were found to be

fairly constant. This will be seen from Table II which gives data for extracts from six other batches of autoclaved lemon juice prepared in a similar manner and examined under the same conditions.

Table II.

Batch	Volume of autoclaved decitrated lemon juice from which extract was obtained cc.	Final volume of solution containing ethereal extract cc.	A cc. of $N/100$ iodine used up by 5 cc. of solution of extract in 5 min. at neutral reaction	B cc. of $N/100$ iodine used up by 5 cc. of solution of extract in 10 min. at alkaline reaction	Ratio A : B
1	250	100	1·0	7·6	1 : 7·6
2	500	100	2·1	16·7	1 : 8
3	500	100	5·1	40·0	1 : 7·8
4	150	100	1·1	8·0	1 : 7·7
5	150	100	0·5	4·4	1 : 8·8
6	150	100	0·9	7·7	1 : 8·5

It has been pointed out earlier that the material extracted by ether from autoclaved juices is not easily or completely redissolved in water but that it is readily soluble in alkalis or alkali carbonates. In cases where this latter fact was made use of in preparing aqueous solutions of the ethereal extracts instead of dissolving them in methyl alcohol or directly in water, the ratio between the amount of iodine absorbed in neutral solution in 5 mins. to that used up in alkaline solution tended to be constant also but at a lower value than in the former case. Solutions of five extracts were prepared by concentrating the ethereal extracts to small volume under reduced pressure, shaking the concentrated ethereal solution with 3 or 4 successive quantities of N Na_2CO_3 (5–7 cc.) neutralising the carbonate solution to p_H 7·0 with H_2SO_4 and removing CO_2 and ether from solution by exhaustion.

The results of the iodimetric examination of these solutions are recorded in Table III.

Table III.

Batch	Volume of autoclaved decitrated lemon juice from which extract was obtained cc.	Final volume of solution containing ethereal extract cc.	A cc. of $N/100$ iodine used up by 5 cc. of solution of extract in 5 min. at neutral reaction	B cc. of $N/100$ iodine used up by 5 cc. of solution of extract in 10 min. at alkaline reaction	Ratio A : B
1	100	100	0·9	4·5	1 : 5
2	100	100	1·7	7·3	1 : 4·3
3	300	50	4·8	24·0	1 : 5
4	300	50	2·1	17·7	1 : 8·4
5	1000	100	3·8	16·0	1 : 4·2

When small amounts of Na_2CO_3 or $NaHCO_3$ were added to the neutral reaction mixtures of iodine and those extracts in purely aqueous solutions, the

amounts of iodine absorbed in 5 min. also increased with the consequent lowering of the ratio to a value approaching that observed above (see Table IV).

Table IV.

Batch	Volume of autoclaved juice or solution from which ethereal extract was obtained cc.	Final volume of solution containing ethereal extract cc.	A_1 cc. of $N/100$ iodine used up by 5 cc. of solution of extract in 5 minutes at neutral reaction	A_2 cc. of $N/100$ iodine used up by 5 cc. of solution of extract in 5 minutes in the presence of 0·5 cc. M $NaHCO_3$	B cc. of $N/100$ iodine used up by 5 cc. of solution of extract in 10 minutes at alkaline reaction	Ratio A_1:B	Ratio A_2:B
Decitrated lemon juice	1000	100	3·6	7·7	28	1:7·8	1:3·6
Solution of 1·0% invert sugar and 0·25% sodium carbonate mixture	1000	100	3·8	8·1	30	1:7·9	1:3·7

If in the reactions in neutral solution between iodine and the extracts which had been dissolved in carbonate solution, mineral acid was added immediately after the end-point was reached in the back titration with sodium thiosulphate, iodine was instantly liberated.

The reactions of phenols with iodine. $M/100$, $M/200$, $M/400$ and $M/1000$ solutions of quinol, catechol, homocatechol and protocatechuic acid were employed in studying the reactions of these polyhydric phenols with iodine. In the experiments in neutral solution using 5 or 10 cc. of these solutions and $N/100$ I in excess, consistent results could only be obtained with quinol. In the case of the more dilute solutions it was found that, providing twice the theoretical amount of iodine was employed, the reaction was strictly quantitative, two atoms of iodine reacting with one molecule of quinol yielding benzoquinone according to the equation

$$C_6H_4(OH)_2 + I_2 \rightleftharpoons C_6H_4O_2 + 2HI.$$

After the end-point in the back titration had been reached iodine separated slowly from these solutions until a condition of equilibrium was attained. The results of these experiments are shown in Table V.

Table V.

Solution of quinol	cc. of $N/100$ iodine used up in 5 min. by 10 cc. of quinol solution at neutral reaction	Theoretical amount of iodine required for reaction involving 1 molecule of quinol and 2 atoms of iodine cc. $N/100$
$M/100$	19·2	20·0
$M/200$	9·6	10·0
$M/400$	5·0	5·0
$M/1000$	2·0	2·0

In the case of catechol, homocatechol and protocatechuic acid the amount of iodine used up was less than that required for the reaction analogous to that between iodine and quinol. When, however, either Na_2CO_3 or $NaHCO_3$ was added to the solutions of these phenols during the reactions, preferably

in amounts molecularly equivalent to the amounts of phenol used, the reactions proceeded quantitatively according to the general equation

$$X.C_6H_3(OH)_2 + I_2 \rightarrow X.C_6H_3O_2 + 2HI$$
$$(X = H, CO_2H \text{ or } CH_3).$$

On acidifying these solutions with mineral acid very soon after the end-point in the back titration, iodine was immediately liberated.

The reactions in alkaline solution were complete in 10 min. and consistent results could be obtained with each phenol. When suitable amounts of iodine were used the quantities of iodine used up were found to bear a quantitative relationship to the amount of phenol employed. The amount of iodoform which separated was very small except in the case of quinol. The results of these experiments are recorded in Tables VI and VII.

Table VI.

Solution of quinol	cc. $N/100$ iodine used up in 10 min. by 10 cc. of quinol solution at alkaline reaction	Theoretical amount of iodine required for reaction involving 1 molecule of quinol and 12 atoms of iodine cc. $N/100$
$M/100$	11·5	12·0
$M/200$	5·7	6·0
$M/400$	2·9	3·0
$M/1000$	1·2	1·2

Table VII.

Solution	Amount of phenol solution used cc.	cc. of $N/100$ iodine used up in 10 min. at alkaline reaction	No. of atoms of iodine per molecule of phenol involved in reaction
$M/100$ catechol	5	3·0	6·0
$M/100$ homocatechol	10	8·0	8·0
$M/100$ protocatechuic acid	10	6·4	6·4

The ratios of the amounts of iodine used up by these phenols in the reaction according to the above general equation to the amounts used up in the presence of caustic alkali are:

quinol	1:6
catechol	1:3
homocatechol	1:4
protocatechuic acid	1:3·2

when the former reaction is quantitative, as in the presence of sodium carbonate or bicarbonate. Mention was made earlier that, in the absence of these salts, this reaction does not proceed quantitatively, except in the case of quinol. It would therefore be expected that in the case of catechol and its derivatives the above minimum values of these ratios would under certain conditions reach a value of 1:8, similar to that obtained with the ethereal extracts from autoclaved decitrated lemon juice and autoclaved solutions of invert sugar and sodium carbonate mixture.

The above experiments show that the general behaviour of these phenols during these reactions is similar to that of solutions of the ethereal extract and that the behaviour of the ethereal extracts approximates to that of catechol and its derivatives rather than to that of quinol. This circumstance coupled with the observation that the ethereal extracts give a catechol colour reaction with ferric chloride solution, among other typical reactions for phenols, suggests that a compound resembling catechol or its derivatives is formed from the sugars in the process of autoclaving.

THE INFLUENCE OF ENZYMES ON THE INACTIVATION OF THE REDUCING PRINCIPLE.

It was observed in connection with the instability of the reducing principle and vitamin C of autoclaved decitrated lemon juice [Zilva, 1929] that the treated medium as well as the original juice was capable of giving a reaction with *p*-phenylenediamine and H_2O_2. In consequence of this observation the existence of a "thermostable peroxidase" which had some influence on the inactivation of the two principles was postulated. An endeavour was now made to co-ordinate this phenomenon with the recent development of the subject.

In the following experiments 5 cc. of the solution to be tested, 1 cc. of 1 % *p*-phenylenediamine, 1 cc. of 1 % benzidine or 1 cc. of 10 % tincture of guaiacum and 4 or 5 drops of 6 % H_2O_2 were used. We found this strength of H_2O_2 convenient since in most experiments the solution contained reducing substances which destroyed this reagent. Blanks were used in all the tests.

Decitrated lemon juice, autoclaved decitrated lemon juice and autoclaved 1 % solutions of sugars were examined and the following facts were observed.

(*a*) They all gave a colour reaction with *p*-phenylenediamine but not with either benzidine or guaiacum tincture.

(*b*) The autoclaved solutions of pure sugars gave a colour reaction with *p*-phenylenediamine immediately, while the remainder responded only after a period of lag which varied from 15 to 40 min. It has been mentioned earlier that the autoclaved solutions of pure sugars as distinct from those containing sodium carbonate *etc.* do not contain iodine-absorbing substances. That this lag period was caused by the inhibiting influence of iodine-absorbing substances was shown by the fact that the lag period was reduced when the iodine-absorbing substances were removed either by precipitation with normal lead acetate (3 cc. of a mixture of 1 volume of saturated normal lead acetate solution and 4 volumes of water per 100 cc. of autoclaved sugar solution) or by adsorption with norite charcoal. In the case of the sugar solutions the iodine-absorbing substances were removed almost completely by the charcoal and the lag period thus reduced to zero. Typical results are shown in Table VIII.

It was found that this lag in the *p*-phenylenediamine-H_2O_2 reaction could be simulated by the addition of catechol to autoclaved solutions of the pure sugars.

(*c*) The ethereal extracts from autoclaved decitrated lemon juice and from an autoclaved solution containing 1 % sugar and 0·25 % sodium carbonate also give the *p*-phenylenediamine-H_2O_2 reaction after a period of lag.

Table VIII.

Solutions examined	cc. of *N*/100 iodine used up in 5 min. at neutral reaction by 5 cc. of autoclaved solution	Time in min. for colour to develop in *p*-phenylene-diamine-hydrogen peroxide test
1. 1·0 % invert sugar solution autoclaved 1 hr. at 143°	0·1	Colour developed immediately
2. Solution of 1·0 % invert sugar and 0·25 % sodium carbonate mixture autoclaved 1 hr. at 143°	7·9	10
3. Solution 2 after treatment with normal lead acetate solution	4·2	5
4. Solution of 1·0 % invert sugar and 0·25 % sodium carbonate mixture autoclaved 1 hr. at 145°	6·9	20
5. Solution 4 after treatment with norite charcoal	0·3	Colour developed immediately
6. Decitrated lemon juice autoclaved 1 hr. at 143°	8·2	15
7. Solution 6 after treatment with norite charcoal	1·9	0·5

(*d*) The ethereal extract from autoclaved decitrated lemon juice, which, as pointed out above, shows "thermostable peroxidase" activity, when added to decitrated lemon juice from which enzymes have been removed by precipitation with alcohol, increases the rate of destruction of the reducing principle when the mixture is stored aerobically at neutral reaction. This is shown by the following experiment. Decitrated lemon juice, p_H 7·0, was concentrated under reduced pressure at 45° to one-fifth of its original volume and was then diluted by the slow addition of 5 times its volume of 96 % alcohol. The filtrate was evaporated to a thin syrup under reduced pressure at 45°. The residue was made up to a convenient volume at p_H 7·0. Such a preparation showed no "peroxidase" activity and usually contained 75 to 80 % of the reducing capacity of the original juice as measured by the reduction of phenolindophenol.

An ethereal extract obtained from 100 cc. of autoclaved decitrated lemon juice and an amount of the above preparation equivalent to 100 cc. of the original juice were mixed and finally made up to 100 cc. at p_H 7·0 and stored in the cold room, together with a control solution of the "peroxidase-free preparation" of the reducing principle. The solutions were titrated at intervals with 0·02 % phenolindophenol. The result of this experiment is shown in Fig. 3.

(*e*) The residue obtained by evaporating under reduced pressure an autoclaved solution of 1·0 % invert sugar and 0·25 % sodium carbonate, which, as has been pointed out previously, contains both iodine-absorbing substances and the "thermostable peroxidase," increases the rate of destruction of the reducing principle in the above "peroxidase-free preparation" on storage in air. Some of the iodine-absorbing substances, which can be obtained free

from the "thermostable peroxidase" by precipitation with normal lead acetate solution produce the same effect without the aid of the "thermostable peroxidase." These observations are based upon the experiments described below in which the following preparations were used.

Preparation A. A solution of 1·0 % invert sugar and 0·25 % sodium carbonate was autoclaved as already described.

Preparation B. 100 cc. of preparation A were boiled for 2 min. with 2 g. of norite charcoal and then filtered. The filtrate was then shaken with 1 g. of charcoal and again filtered. The latter process was repeated giving a final almost colourless solution.

Preparation C. 3 cc. of normal lead acetate solution (one volume of saturated normal lead acetate solution mixed with four volumes of water) were

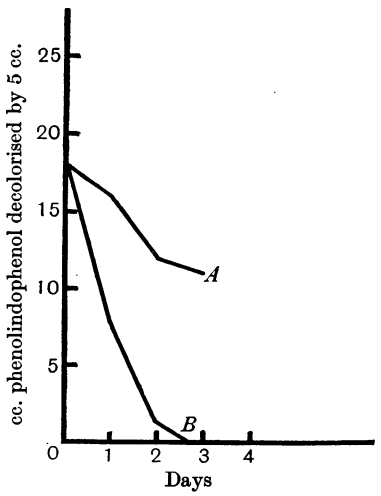

Fig. 3. *A*, "peroxidase-free preparation" of reducing principle; *B*, "peroxidase-free preparation" of reducing principle containing ethereal extract from autoclaved decitrated lemon juice.

added to 100 cc. of solution A and the precipitate was centrifuged off. This precipitate was suspended in 20 cc. of water and decomposed with 0·2 cc. of 4*N* H_2SO_4. The lead sulphate was removed on the centrifuge. The clear liquid was adjusted to p_H 7·0 and then diluted to 50 cc. with water.

Preparation D. The lead was removed as lead sulphate from the filtrate obtained in preparation C after precipitation with lead acetate. The solution was finally adjusted to p_H 7·0.

The preparations A, B, C and D were examined iodimetrically and for "thermostable peroxidase" with 1 % *p*-phenylenediamine solution and H_2O_2. The details are given in Table IX. (Solution C was diluted to half strength before samples were taken for test.)

Preparation E. A "peroxidase-free preparation" of the reducing principle was prepared as above from 400 cc. of decitrated lemon juice. The final solution measured 100 cc.

50 cc. of solutions A, B and D were concentrated under reduced pressure at 45°. Each was then mixed with 20 cc. of solution E, as was also 25 cc. of solution C and then each diluted to 50 cc. at neutral reaction. These solutions, together with 20 cc. of solution E diluted to 50 cc. with water as a control, were stored aerobically in the cold room and titrated periodically with 0·02 % phenolindophenol.

The results of this experiment are recorded in Fig. 4.

Table IX.

Preparation	cc. of $N/100$ iodine used up by 5 cc. of preparation in 5 min. at neutral reaction	Time in min. for colour to develop in *p*-phenylene-diamine-hydrogen peroxide test
A	4·9	10
B	0·3	Colour developed immediately
C	1·7	No development of colour occurred in 1 hour
D	2·2	3

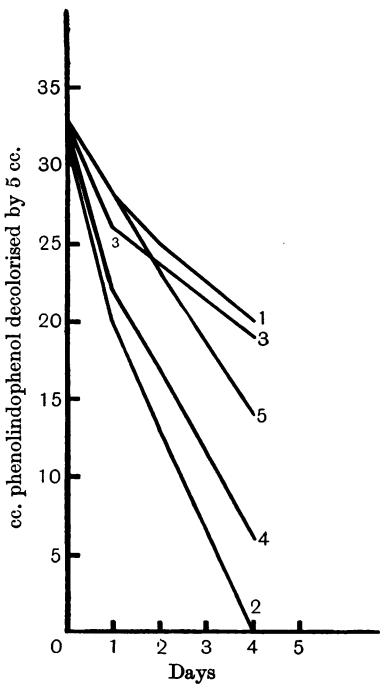

Fig. 4. 1, "peroxidase-free preparation" of reducing principle; 2, "peroxidase-free preparation" of reducing principle containing preparation A; 3, "peroxidase-free preparation" of reducing principle containing preparation B; 4, "peroxidase-free preparation" of reducing principle containing preparation C; 5, "peroxidase-free preparation" of reducing principle containing preparation D.

(*f*) Quinol, catechol, homocatechol and protocatechuic acid in 0·05 % concentration, whether alone or in the presence of the "thermostable peroxidase", do not increase the rate of destruction of the reducing principle in the above described "peroxidase-free preparation" of decitrated lemon juice

on storage in air whereas benzoquinone in this concentration destroys the reducing principle almost immediately under the same conditions. On the other hand these phenols accelerate the destruction of the reducing principle in this preparation in the presence of a true peroxidase, as for example that from turnip root, at about the same rate at which they increase the rate of its inactivation in decitrated lemon juice.

In these experiments 600 cc. of a "peroxidase-free preparation" of the reducing principle were prepared and one-half was used to dissolve the residue from 300 cc. of a 1 % solution of invert sugar which had been autoclaved as previously described. The residue, which contained the "thermostable peroxidase" but, as mentioned earlier, no iodine-absorbing substances, was obtained by evaporation under reduced pressure at 45°. 0·025 g. of quinol, catechol, homocatechol and protocatechuic acid were separately dissolved in 50 cc. of each of the above preparations. A "peroxidase-free preparation" of the reducing principle containing 0·05 % of benzoquinone was also included in this experiment. The solutions were then adjusted to p_H 7·0 and stored aerobically in the cold room. They were titrated at intervals with 0·02 % phenolindophenol. Table X records the results obtained.

Table X.

Solutions	cc. of 0·02 % phenolindophenol solution decolorised by 5 cc. of test solution			
	1st day	3rd day	5th day	8th day
1. "Peroxidase-free" preparation of the reducing principle of decitrated lemon juice	22	19	17	14
2. Solution 1 containing 0·05 % of quinol	22	18	16	13
3. ,, ,, catechol	22	20	18	16
4. ,, ,, homocatechol	22	20	18	16
5. ,, ,, protocatechuic acid	22	22	17	15
		2nd day		
6. ,, ,, benzoquinone	30	0	—	—

Solutions	cc. of 0·02 % phenolindophenol solution decolorised by 5 cc. of test solution			
	1st day	3rd day	5th day	8th day
1 A. "Peroxidase-free" preparation of the reducing principle of decitrated lemon juice containing "thermostable peroxidase"	22	18	17	14
2 A. Solution 1 A containing 0·05 % of quinol	22	16	14	12
3 A. ,, ,, catechol	22	17	16	15
4 A. ,, ,, homocatechol	22	16	14	14
5 A. ,, ,, protocatechuic acid	22	22	15	14

One-half of a "peroxidase-free preparation" of the reducing principle obtained from 400 cc. of decitrated lemon juice was mixed with 75 cc. of a solution of turnip root peroxidase and the mixture diluted to 150 cc. The preparation of the peroxidase solution is given below. The other half was diluted with water to 150 cc. In 50 cc. of each solution were dissolved 25 mg.

of quinol and 25 mg. of catechol respectively. The remainder served as controls. These solutions were then adjusted to neutral reaction and stored aerobically in the cold room. They were titrated periodically in the usual manner. Fig. 5 shows the results of these experiments. The peroxidase was still very active in all the solutions at the end of the experiments.

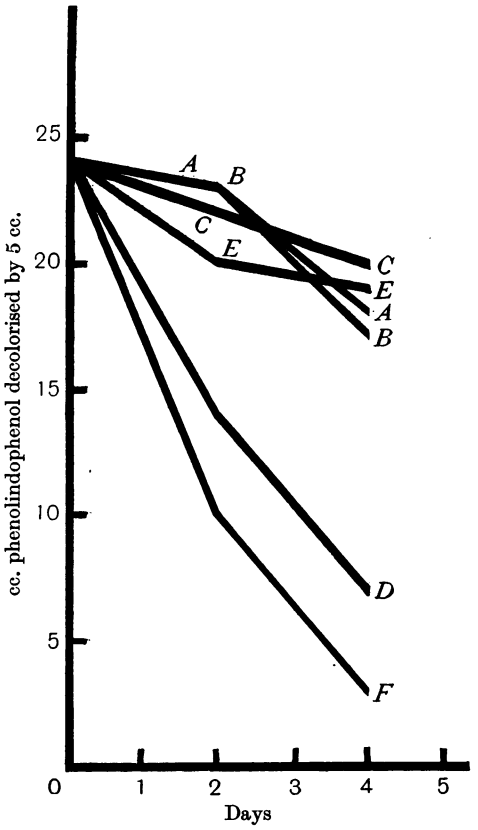

Fig. 5. *A*, "peroxidase-free preparation" of reducing principle; *B*, "peroxidase-free preparation" of reducing principle containing added turnip peroxidase; *C*, "peroxidase-free preparation" of reducing principle containing 0·05 % catechol; *D*, "peroxidase-free preparation" of reducing principle containing added turnip peroxidase and 0·05 % catechol; *E*, "peroxidase-free preparation" of reducing principle containing 0·05 % quinol; *F*, "peroxidase-free preparation" of reducing principle containing added turnip peroxidase and 0·05 % quinol.

The solution of the peroxidase was obtained from a frozen turnip root weighing 300 g. by removing the outer cortex while the root was still frozen and then slicing and mincing the remainder so that the pulp fell directly into an excess of alcohol in which it was allowed to thaw. The enzyme was then obtained by extracting the alcohol-free pressed tissue residue with water, the extracts being finally diluted to 200 cc. This enzyme preparation, which was neutral in reaction, gave no colour reaction with ferric chloride solution, did not reduce ammoniacal silver nitrate solution, Fehling's solution or phenol-

indophenol solution or absorb iodine from neutral solutions even after long standing. It was therefore considered to be free from phenolic substances. Four drops of this solution in 5 cc. of water gave very intense peroxidase reactions with 1 cc. of 1·0 % solutions of *p*-phenylenediamine, benzidine and 10 % tincture of guaiacum and H_2O_2.

(*g*) Although decitrated lemon juice does not contain a true peroxidase it seems to contain a thermolabile factor which catalyses the destruction of the reducing principle of the juice in the presence of polyhydric phenols. Thus when decitrated lemon juice at p_H 7·0 was steamed in ampoules under strictly anaerobic conditions for 1 hour the *p*-phenylene-diamine-H_2O_2 test was still given, as was to be expected, by this heated juice though the lag period had increased. 0·05 % of catechol or quinol was dissolved in such steamed juice and the solutions stored. Periodic titrations with phenolindophenol solution showed that the reducing principle was not destroyed at a greater rate in these heated juices containing the phenols, as it is in the case of unheated juice.

Steaming decitrated lemon juice under these conditions appears not to destroy the reducing principle to any appreciable extent nor is there any change in the iodine-absorbing capacity or in the condition of the reducing sugar.

The results of these experiments are shown in Fig. 6 and Table XI.

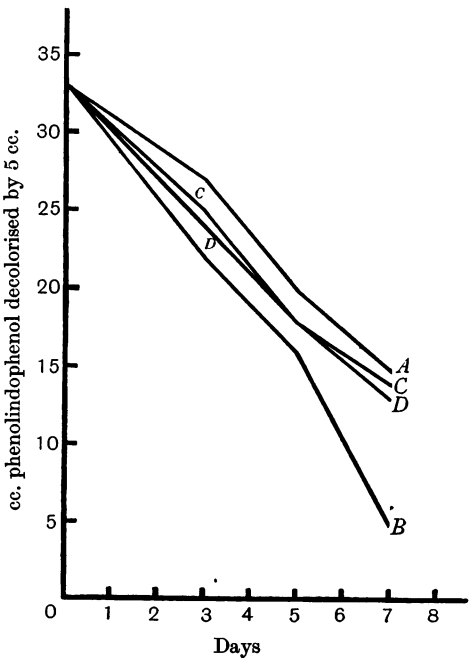

Fig. 6. *A*, decitrated lemon juice; *B*, decitrated lemon juice containing 0·05 % quinol; *C*, decitrated lemon juice steamed 1 hour; *D*, decitrated lemon juice containing 0·05 % quinol steamed 1 hour.

Table XI.

Solution	cc. of 0·02 % phenolindophenol solution decolorised by 5 cc. of juice	Amount of iodine expressed in cc. of N/100 sclution used up by 5 cc. of juice in 5 minutes at neutral reaction	Amount of reducing sugar expressed in mg. of glucose contained in 5 cc. of juice determined by Bertrand's method
Decitrated lemon juice	23	5·8	63
Decitrated lemon juice after heating in steamer for 1 hr.	21	5·7	63

Discussion.

The foregoing results prove clearly that the substance formed on autoclaving decitrated lemon juice which accelerates the inactivation of the reducing principle and of vitamin C on storage of the juice at neutral reaction originates from the sugar of the decitrated lemon juice. Further, the evidence obtained points rather convincingly to the fact that this substance is of the nature of a catechol compound. It is difficult to assert with precision from the available data the manner in which this substance actually inactivates the vitamin complex. We know however from observations described in earlier communications of this series that when quinol, catechol, resorcinol, benzoquinone or quinhydrone is added to ordinary unheated decitrated lemon juice, the same inactivating action can be obtained and it is therefore evident that this effect is produced by a great number of chemically related substances.

With regard to the "thermostable peroxidase," which was previously considered to be associated with the acceleration of the inactivation of the reducing principle and vitamin C [Zilva, 1929], the bulk of the evidence in this investigation indicates that this factor plays no part in the inactivating mechanism. Much more significant in this respect is the observation made that in decitrated lemon juice the reducing principle is destroyed on the addition of catechol and quinol whilst this destruction does not take place if the juice has been previously heated for 1 hour in a steamer. It is further of interest to note here that the dihydric phenols require the aid of some factor of an enzymic nature to effect this inactivation, whilst benzoquinone and the ethereal extracts of autoclaved decitrated lemon juice cause it unaided. The only reasonable assumption we may therefore make at this stage is that the phenolic hydroxy-compounds undergo intermediate oxidation and subsequently oxidise the vitamin complex.

Summary.

1. When the sugar of decitrated lemon juice is removed by anaerobic fermentation, the reducing principle and the antiscorbutic factor in the fermented juice are not inactivated at an increased rate after autoclaving and storing at neutral reaction, as they are in autoclaved unfermented decitrated lemon juice on storage.

2. Solutions of glucose, fructose or invert sugar containing certain salts, on being autoclaved under the same conditions as decitrated lemon juice, are

capable of increasing the rate of destruction of the reducing principle and vitamin C in decitrated lemon juice in neutral solution.

3. The chemical properties of the ethereal extracts of autoclaved decitrated lemon juice and of autoclaved solutions of sugar-salt mixtures have been studied. Both extracts are capable of accelerating the destruction of the reducing principle and vitamin C in decitrated lemon juice on storage.

4. It is concluded that the substance or substances which conduce to the acceleration of the inactivation of the above principle are produced from the sugar of the juice on autoclaving.

5. From a comparative study of the chemical properties of the ethereal extracts of autoclaved decitrated lemon juice and autoclaved sugar solutions with those of polyhydric phenols, evidence was obtained that the substance or substances formed from the sugars on autoclaving are most probably related to catechol.

6. On autoclaving decitrated lemon juice or solutions containing the same sugar concentrations a substance is produced which is capable of oxidising *p*-phenylenediamine but not guaiacum or benzidine.

7. Catechol and quinol do not destroy the reducing principle in decitrated lemon juice which has been steamed anaerobically for 1 hour, whilst they do so in the unheated juice.

8. These dihydric phenols are unable to destroy the reducing principle in the absence of an enzymic factor, whilst benzoquinone and the ethereal extract do so unaided.

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