both from experiments in which the exact minimum protective dose is found and from those in which there is a definite partial protection to which a quantitative rating can be given.   This method possesses the advantage of the method of minimum protective doses, and in addition permits the use of a numerical value for each individual experiment of a series, the average of which should yield a more trustworthy result than when only those animals receiving exactly the minimum protective dose are taken into account.   The data presented in the paper which follows will illustrate the application of this method.

Summary.

   Relative amounts of antiscorbutic vitamin are measured by determining how much of the food under test is required to prevent scurvy in guinea pigs, or by a quantitative rating of the severity of the scurvy produced when less than this "minimum protective dose" is fed.   This rating is based upon the weight-curve, duration of life, symptoms, and especially the autopsy findings of guinea pigs of standard initial age and weight.   A new basal diet designed to furnish optimum quantities of all essential nutrients other than the antiscorbutic vitamin, and the technique of the experiments, are described.

   NEW YORK, N. Y.

THE EFFECT OF TEMPERATURE AND THE CONCENTRATION OF HYDROGEN IONS UPON THE RATE OF DESTRUCTION OF ANTISCORBUTIC VITAMIN (VITAMIN C).[1,2]

BY V. K. LAMER, H. L. CAMPBELL AND H. C. SHERMAN.

   While the great instability of the antiscorbutic vitamin (Vitamin C) makes it an unpromising material for attempts at actual isolation, the development of methods for measuring relative amounts of this substance with considerable accuracy makes possible the study of its chemical behavior.   The present investigation deals with the effects of heating for different lengths of time at 60°, 80°, and 100°, and at different known concentrations of hydrogen ion.   As indicated by the earlier work of Delf,[3] the reaction by which Vitamin C is destroyed has a measurable

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[1] The data for this paper are taken from a Dissertation submitted by Victor K. LaMer for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University, June, 1921.   For the sake of brevity many of the details included in the dissertation are here omitted.

[2] Preliminary reports were presented at the January meeting of the Society of Experimental Biology and Medicine (*Proc. Soc. Exptl. Biol. Med.*, **18**, 122 (1921)) and the Rochester meeting of the American Chemical Society, April, 1921.

[3] Delf, *Biochem. J.*, **12**, 416 (1918).

velocity, which velocity is accelerated by rise in temperature though less rapidly than most chemical reactions.

The experiments of Harden and Zilva,[4] and Hess and Unger[5] have also shown that the addition of alkali to orange juice reduces the stability of the antiscorbutic vitamin.

In view of the fact that in a later paper Delf[6] states "that neutralizing nearly all of the excess acid in orange juice therefore does not appreciably reduce the stability of the antiscorbutic constituent of the juice at 100°," it was considered that an investigation of the rate of destruction at various measured hydrogen-ion concentrations under definite conditions of time and temperature of heating would be desirable.

In the experiments described it is believed that the influence of these factors has been measured quantitatively with a probable error of less than 5% for each of the chief points determined.

Selection and Preparation of Antiscorbutic Materials.

The juice from canned tomatoes was selected as the source of Vitamin C in this investigation for the following reasons.

(1) It contains a high concentration of this vitamin, 3 cc. sufficing to give complete protection to a guinea pig, which is an important point in quantitative feeding work since it permits one to reduce the concentration of active material to 10% or even less of its original value and yet not require the administration of an excessive volume in order to attain the same degree of protection as the untreated product affords.

(2) It is possible to eliminate in a large measure the variation in initial antiscorbutic content due to seasonal or other causes, by purchasing the canned tomatoes in case lots.

Preparation of Materials.—The material used in feeding was prepared at 5-day intervals by opening one or more cans of tomatoes, and filtering at 10°, first through muslin and then through filter paper. The filtrate, which possessed at most only a slight opalescence, was measured into Erlenmeyer flasks made of Pyrex glass, heated rapidly over a wire gauze with constant swirling until it had attained the desired temperature (which required 1 to 2 minutes) and then at once put into a constant temperature bath accurate to ±0.2°. The evaporation which occurred through the cotton stoppers was noted and either replaced by addition of sterile water, or a correction for the concentration resulting thereby was made in calculating the dosage of the heated juices.

At the end of the heating period, the flasks were cooled quickly (1 to 2 minutes) by means of running water and then placed on ice. All anti-

[4] Harden and Zilva, Lancet, II, 320 (1918).
[5] Hess and Unger, J. Biol. Chem., 38, 297 (1918).
[6] Delf, Biochem. J., 14, 220 (1920).

scorbutic material, whether in cans or flasks, was kept at 10° at all times, except for the period of heating indicated. Bacterial action was very carefully avoided.

In all, 4 lots of canned tomatoes were used, each of which was standardized independently, 23 animals being used for this purpose.

In order to test the influence of hydrogen ions or their reciprocal, hydroxyl ions, samples of tomato juice of different degrees of acidity or alkalinity were prepared. The normal hydrogen-ion concentration of the juice from different lots was found to lie between $10^{-4.18}$ and $10^{-4.37}$. When the juice was heated in a boiling water-bath for an hour the hydrogen-ion concentration remained practically unchanged, namely $10^{-4.18}$ before, to $10^{-4.23}$ after heating. When the samples were made slightly alkaline difficulty was encountered in maintaining a constant hydrogen-ion concentration due to the fact that at 100° the sugars present are attacked by the hydroxyl ions, yielding organic acids, as the work of Nef[7] has shown, which tends to reduce the alkalinity. A certain amount of discoloration occurred which is very likely due to caramelization of the sugars. Table I shows these effects.

The preparations designated as I, II, III were made by adding the amount of sodium hydroxide indicated in Table I, or its equivalent, per 100 cc. of freshly filtered tomato juice, and heating for 1 hour at 100°. When the period of heating had expired the preparations were at once cooled under the tap in exactly the same manner as the preparations to which no alkali had been added. An amount of hydrochloric acid equivalent to the sodium hydroxide was next added to neutralize the latter so that the effects of the addition of alkali would take place only during the period of heating. Inasmuch as previous workers have not re-acidified after heating the alkaline preparations it was thought advisable to determine whether this might have any influence upon the results.

Preparation III was accordingly divided into two parts at the conclusion of the heating period; IIIa being re-acidified as usual, while in the case of IIIb the preparation was allowed to remain in its alkaline condition at the usual temperature of 10° for the period of 1 to 5 days, during which it was administered daily to the animals of that series.

The changes in volume due to the addition of acid or alkaline solutions were taken account of in all calculations. The hydrogen-ion concentrations were determined electrometrically with the apparatus described by Thomas and Baldwin.[8]

[7] Nef, *Ann.*, **403**, 204 (1914).

[8] Thomas and Baldwin, THIS JOURNAL, **41**, 1990 (1919).

TABLE I

HYDROGEN-ION CONCENTRATIONS OF PREPARATIONS TO WHICH SODIUM HYDROXIDE
HAS BEEN ADDED

Prep. No.	0.2 N NaOH per 100 cc. of tomato juice Cc.	Log. of C_{H^+}	Remarks
I	11.0	− 5.20	before heating.
I	11.0	− 4.88	after heating 1 hr. at 100°.
II	37.0	− 9.14	before heating.
II	37.0	− 7.53	after heating 1 hr. at 100°.
III (a,b)	74.0	−10.88	before heating.
III (a,b)	74.0	− 8.26	after heating 1 hr. at 100°.

The solutions described were fed to experimental animals (guinea pigs) and the percentage destruction of the vitamin measured by the method described in the preceding paper[9] with the average results shown in Tables II and III.

TABLE II

THE DESTRUCTION OF ANTISCORBUTIC VITAMIN AT HYDROGEN-ION CONCENTRATION EQUAL TO $10^{-4.18}$ TO $10^{-4.37}$ (NATURAL REACTION OF TOMATO JUICE). TIME AND TEMPERATURE VARIED.

No. of expts.	Time Hours	Temp. °C.	Average destruction %
12	1	100	50.2±1.3[a]
3	2	100	58. (about)
11	4	100	67.7±0.9[a]
6	1	80	40.5±1.2[a]
5	4	80	53.0±1.3[a]
5	1	60	25.2±1.0[a]
6	4	60	36.8±1.5[a]

[a] See discussion following on probable errors.

TABLE III

DESTRUCTION OF ANTISCORBUTIC VITAMIN IN TOMATO JUICE AT VARIOUS HYDROGEN-ION CONCENTRATIONS. SODIUM HYDROXIDE SOLUTION ADDED AND MATERIAL HEATED FOR 1 HOUR AT 100°, FOLLOWED BY RE-ACIDIFICATION WITH HYDROCHLORIC ACID AT EXPIRATION OF HEATING PERIOD, EXCEPT IN THE CASE OF PREPARATION IIIB WHICH WAS ALLOWED TO REMAIN ALKALINE AFTER HEATING AND FED OVER A PERIOD OF 5 DAYS. ALL PREPARATIONS WERE PRESERVED AT 10°.

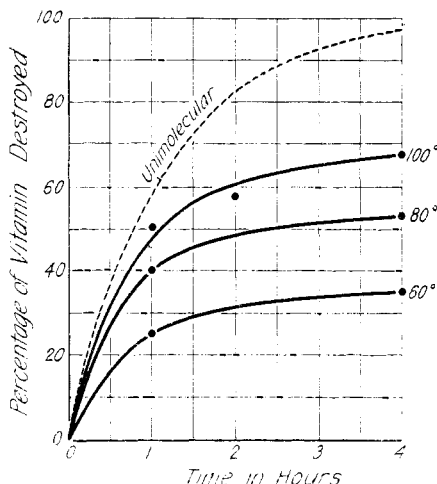| Prep.	No. of expts.	Log. C_{H^+} before heating	Log. C_{H^+} after heating	Destruction %
Natural reaction	12	− 4.3	−4.3	50.2±1.3[a]
I	4	− 5.2	−4.9	58.3±0.7[a]
II	5	− 9.2	−7.5	61.8±0.8[a]
IIIa	2	−10.9	−8.3	61–65 (about)
IIIb	3	−10.9	−8.3	90–95 (about)

[a] See discussion following on probable errors.

[9] Sherman, LaMer and Campbell, THIS JOURNAL, **44**, 165 (1922).

Discussion of Results.

Errors Involved.—The experimental errors in the temperature of heating ($\pm 0.2°$) are negligible, inasmuch as the temperature coefficient as shown later is of the low order of 1.2. The maximum possible error in time of heating could not exceed 4 minutes in 60, or 6.6%; and where this procedure is repeated as many as 18 times for a single experiment the probable error through laws of chance should be very much less. In the case of 4 hours' heating the effect of this error is still further reduced. The error in feeding was of the order of 2 to 3%. The largest error involved in the work is that due to variation of experimental animals. Inspection of the individual data[10] shows that the maximum variation for single experiments is about 15%. The average of these data and the numbers of experiments are given in Tables II and III. The "probable errors" given were calculated by the usual formula, but it should be stated that they are included only for the purpose of giving an idea of the degree of variation in the results of a given series, since in some of the series the number of experiments was not sufficient to warrant giving them the strict interpretation of a probable error. The general concordance of the values given, however, would indicate that the probable error of the average due to animal variation is less than 5%.

HEAT DESTRUCTION OF ANTISCORBUTIC VITAMIN OF TOMATO JUICE AT NATURAL ACIDITY ———— $C_H{}^+ = 10^{-4.3}$

The form of the time curve of destruction.—When the percentage destruction from Table II is plotted against the time as we have done in Fig. 1, it is at once apparent that the destruction of the antiscorbutic vitamin is not dependent upon any one critical temperature, but that the degree of destruction is a function of both the time and the temperature. Further examination shows that it is not possible to express the relationship between these two factors by any of the simple equations of chemical kinetics. If an attempt is made to fit a curve of the unimolecular type it is evident that there is a very marked tendency for the curve to flatten with the progress of time. At $100°$ the values for k in the equation

$$\frac{1}{t} \log\left(\frac{1}{1-x}\right) = k$$

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[10] Table V, Ref. 1, Dissertation.

(where x is the fraction changed at the time t) are 0.303 at 1 hour, 0.187 at 2 hours, and 0.123 at 4 hours.

Nor do the curves follow the square-root law of Schutz, for in the latter case the destruction at 4 hours should be double that at 1 hour, but instead the destruction seems to be more nearly a function of the 4th root of the time. Thus when we calculate K by substituting the data from Table III in the equation

$$K = \frac{x}{\sqrt[4]{t}} \quad \text{(where } x \text{ and } t \text{ have the significance given above)}$$

we find values which are very nearly constant for the different temperatures.

Thus $K_{100}°$ 1 hour, 0.50; 2 hours, 0.49; 4 hours, 0.48. Av. $K_{100}°$, .49. $K_{80}°$ 1 hour, 0.40; 4 hours, 0.38. Av. $K_{80}°$, 0.39. $K_{60}°$ 1 hour, 0.25; 4 hours, 0.26. Av. $K_{60}°$, 0.255.

It was considered at the outset that it was more important for the purposes of this investigation to obtain reliable representative data at different temperatures than it was to obtain a great many data at any one temperature, and for that reason we have tried to establish only two points on a given temperature curve but to establish these as accurately as possible by the use of an adequate number of animals.

It is interesting to note that this flattening of the reaction curve is frequent also in reactions catalyzed by enzymes such as the hydrolysis of lactose by lactase,[11] the digestion of casein[12] by trypsin, and the action of amylases on starch.[13]

The temperature coefficient.—Using the values of K obtained above for substitution in the equation

$$Q_{10} = \sqrt{\frac{K_{t+20°}}{K_t}}$$

we obtain the following values for the temperature coefficient for the intervals for 60° to 80°, and 80° to 100°.

$Q_{10}(60° \text{ to } 80°) = 1.23$ $\qquad\qquad$ $Q_{10}(80° \text{ to } 100°) = 1.12.$

Delf[3] in her paper upon the destruction of the antiscorbutic vitamin in cabbage leaves offers the value of 1.3 for Q_{10} from 60–100° as a rough approximation made on the assumption that the velocity is uniformly accelerated throughout its course. As far as the authors are aware this is the only statement in the literature regarding the quantitative relation between the temperature and the velocity of the reaction.

[11] Armstrong, *Proc. Roy. Soc.*, (London), **73**, 500 (1904).

[12] Bayliss, "Nature of Enzyme Action," Longmans, Green and Co., 4th Ed., p. 81, for this and other similar work.

[13] Sherman and J. C. Walker, This Journal, **39**, 1476 (1917).

Delf considers this low temperature coefficient to be evidence that the vitamin is neither an enzyme nor a protein, for the temperature coefficients of the inactivation or denaturation of such substances have been found to be extremely high, $i.$ $e.$, of the order $Q_{10}=7$ (heat destruction of emulsin,[14]) to 635 (coagulation of egg albumin,[15]). We agree fully with this view and would also point out that the temperature coefficient observed corresponds almost exactly with that of a diffusion[16] process.

This fact coupled with the fact that tomato juice is of a colloidal (2-phased) nature furnishes presumptive evidence, at least, that in the heat destruction of antiscorbutic vitamin we are dealing with a heterogeneous reaction.[17]

Reactions having temperature coefficients of this order (1.2) are sometimes explained on the ground that a part of the reaction is photochemical in nature. Experiments in which the material was heated at 60° for 1 hour and exposed at the same time to the light from 4 adjacent 500-watt heater lamps showed no increase in destruction over their controls. This is in harmony with Zilva's[18] negative results in which the ultra-violet rays from a quartz mercury lamp were used.

It may be properly asked whether or not the flattening of the reaction curve can be explained on the basis of the tendency to reach an equilibrium point due to the mass-action effect of the products on the velocity of the reverse action, as has been shown to be the case in the hydrolysis of lactose and for the system glycerol : glucoside : emulsin investigated by Dietz.[19]

The principal objection to the acceptance of this point of view lies in the fact that the equilibrium point if there is one must be capable of being shifted from say 75% to 95% of completion by the simple addition of dilute sodium hydroxide causing a change in hydrogen·ion concentration from $10^{-4.3}$ to about 10^{-10}. See Table III.

Our results would warrant the general view that for short periods of time (1 hour or less) the extent of the deleterious influence of heating depends more upon the length of time than it does upon the intensity of the process, while for longer periods (4 hours or more) the effect of the intensity assumes greater prominence in comparison to the time than it did in the shorter period. Thus on heating for 30 minutes, it would

[14] Tammann, $Z.$ $physik.$ $Chem.$, **18**, 426 (1895).
[15] Chick and Martin, $J.$ $Physiol.$, **40**, 404 (1910).
[16] Oholm ($Z.$ $physik.$ $Chem.$, **50**, 309 (1905)) found the temperature coefficient of the diffusion of the common salts, acids and bases to lie between 1.19 and 1.28.
[17] See Lewis, "System of Physical Chemistry," Longmans, Green and Co., 2nd Ed., Vol. 1, Chap. X; and Bayliss, Ref. 12, for a discussion of the kinetics of heterogeneous reactions.
[18] Zilva, $Biochem.$ $J.$, **13**, 164 (1919).
[19] Dietz, $Z.$ $physiol.$ $Chem.$, **52**, 279 (1907).

appear from Fig. 1 that between 27% and 30% of the vitamin is destroyed, whether at 80° or 100°, whereas at the end of 4 hours' heating, the differences are more pronounced for the same temperatures, namely, 53% and 68%.

Effects of decreased hydrogen-ion concentration.—The effects of changed hydrogen-ion concentration are in accordance with the view generally held, since the publication of Holst and Frölich's work, that the vitamin is more stable in acid than in neutral or alkaline solution. The data indicate that neutralization of less than $1/2$ of the natural acidity (bringing the solution to a hydrogen-ion concentration of $10^{-5.2}$) produces a significant change in the velocity of destruction; namely 58% is destroyed instead of 50%. When the tomato juice is first made distinctly alkaline to phenolphthalein with carbon dioxide-free sodium hydroxide, and after heating one hour at 100° is re-acidified with an amount of hydrochloric acid equivalent to the alkali originally added, we find that the destruction is increased to 61%.

On the other hand when we omit the re-acidification (Prep. IIIb) we get a very much greater effect, due in all probability to the continued destructive action of the hydroxyl ions at 10°. The latter result is in accord with the work of Harden and Zilva[4] who found that orange juice made 0.05 N alkaline to phenolphthalein (our Preparation III was 0.0244 N alkaline) gave partial protection when fed the same day, but no protection after standing for 24 hours. Hess and Unger[5] report similar results, but neither investigator states the exact temperature employed.

Oxidation and Reduction.—Following the completion (March, 1921) of the experimental work given in the preceding tables, 5 papers have appeared in rapid succession dealing with the effect of various oxidizing agents upon the destruction of Vitamin C.[20, 21, 22, 23, 24] The data given show that drastic oxidizing agents such as potassium permanganate and hydrogen peroxide destroy more or less vitamin at room temperature, and that when de-citrated lemon juice is aerated at 100° for 2 hours the destruction is marked. From these papers some have gained the impression that the destruction process is of a purely oxidative nature and that the sole effect of heat is to increase the rate of this oxidation reaction. In order to test this point guinea pigs were fed doses of 2, 3 and 4 cc. of tomato juice, at hydrogen-ion concentrations of $10^{-4.3}$ and $10^{-8.3}$, into which oxygen had been bubbled at atmospheric pressure while it was being heated at 100° for 1 hour. Other preparations were treated in a like manner except that hydrogen was bubbled through them instead of oxygen. The

[20] Hess, *Proc. Soc. Exptl. Biol. Med.*, **18**, 143 (1921).

[21] Zilva, *Lancet*, I, 478 (1921).

[22] Ellis, Steenbock and Hart, *J. Biol. Chem.*, **46**, 367 (1921).

[23] Anderson, Dutcher, Eckles and Wilbur, *Science N. S.*, **53**, 446 (1921).

[24] Dutcher, Harshaw and Hall, *J. Biol. Chem.*, **47**, 483 (1921).

preliminary results obtained from 13 animals indicate complete destruction in both the acid and alkaline preparations in which oxygen was used. Where hydrogen was used the degree of destruction was greater than in the experiments quoted in Tables II and III, which would support the contention that the destructions measured in those tables are primarily those of a heat effect *per se* and not of atmospheric oxidation. That destruction by atmospheric oxygen could have played only a minor rôle in the experiments tabulated above becomes more apparent when we consider that the amount of dissolved oxygen available for such a reaction must of necessity be considerably reduced since it is in equilibrium with a gaseous mixture immediately above the liquid in which the air originally present has been displaced by the water vapor coming from the nearly boiling solution, a condition radically different from the case where oxygen or air is bubbled directly into the hot liquid.

It is entirely possible that the heat destruction of Vitamin C is of the nature of an intramolecular oxidation and reduction such as occurs when aldehydes or sugars are heated, especially in alkaline solution. The addition of external oxidizing or reducing agents then would serve to aid in the speed and completeness of the reaction. The writers intend, when more data are available, to discuss the question of vitamin oxidation from the standpoint of oxidation and reduction potentials, particularly in regard to the effect of hydrogen-ion concentration upon the magnitude of these potentials.

Summary.

1. The effect of heating upon the destruction of the antiscorbutic vitamin in tomato juice has been measured quantitatively ($\pm 5\%$) for periods of 1 to 4 hours, at 60°, 80° and 100°.

2. The results show that the velocity of destruction, under the conditions of the experiments, decreases with the time and in greater degree than would be expected if the reaction followed the unimolecular law or the square root rule of Schutz. The percentage destroyed varied empirically as the 4th root of the time.

3. The temperature coefficient is of low order:
$$Q_{10}\ (60\text{--}80°) = 1.23;\ Q_{10}\ (80\text{--}100°) = 1.12.$$
The reaction is not appreciably sensitive to ordinary light. It is probably of the heterogeneous type.

4. The effect of reducing the hydrogen-ion concentration from C_{H^+} $10^{-4.3}$ to C_{H^+} ($10^{-5.2}$ to $10^{-4.9}$) is to increase the destruction during 1 hour at 100° from 50% to about 58%. When the material was made faintly alkaline, C_{H^+} ($10^{-10.9}$ to $10^{-8.3}$), the destruction was 61–65%.

5. When re-acidification after heating in alkaline solution (C_{H^+} $10^{-10.9}$

to $10^{-8.3}$) was omitted, and the material allowed to remain alkaline at 10° and fed over a period of 5 days, the destruction rose to 90–95%.

6. The possibility of oxidation and reduction in these and other experiments is discussed.

NEW YORK CITY.

THE PREPARATION AND PROPERTIES OF SEVERAL PHENYL ALKYL SUCCINIC ACIDS.

BY FRED W. UPSON AND T. J. THOMPSON.[1]

The original purpose of this investigation was the separation of the optical isomers of *iso*propyl-phenyl-succinic acid prepared by Avery and Upson.[2] To the present time this has not been accomplished although resolution was attempted with brucine, quinine, strychnine and cinchonine. During the course of the attempted resolutions, however, a number of incidental questions arose. Among these were the preparation of other substituted succinic acids, the difficulty of saponification of the nitrils of certain substituted succinic acids already referred to by Avery and Upson, and the structure of the sodium benzyl compound which results from the interaction of sodium amide and benzyl cyanide.

I. Preparation of Nitrils.

Preparation of alkyl phenyl-succino-nitril and of alkyl phenyl-succino-half-nitril half-ester was carried out by condensing alkyl cyanohydrines with benzyl cyanide by means of (1) sodium ethoxide, or methoxide and (2) esters of α-bromo fatty acids with benzyl cyanide by means of sodamide, respectively.

1. **Alkyl Aldehyde Cyanohydrines with Benzyl Cyanide. Discussion.** —When it was determined that *iso*propyl-phenyl-succinic acid could not be resolved, an attempt was made to prepare other substituted succinic acids, in the belief that optically active compounds might be resolved from them. The method of condensation employed was a modification of the one used by Avery and Upson[2] in the preparation of *iso*propyl-phenyl-succino-nitril. The condensations of the cyanohydrines of acetic aldehyde, *n*-propionic aldehyde, acetone, methylethyl-ketone and *iso*valeric aldehyde with benzyl cyanide were attempted in the order mentioned; but *iso*valeric-cyanohydrine was the only one that gave a condensation compound. The yield was very good in the case of the *iso*valeric -phenyl-succino-nitril; also in the case of *iso*propyl-phenyl-succino-nitril. As yet we

[1] The dissertation upon which this paper is based was presented by T. J. Thompson as partial fulfilment of the requirements for the degree of Doctor of Philosophy in the University of Nebraska.

[2] Avery and Upson, THIS JOURNAL, **30**, 600 (1908).