A STUDY OF THE DECARBOXYLATION OF DEHYDROASCORBIC ACID

by

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A THESIS

Presented to the Department of Biochemistry
and the Graduate Division of the University of Oregon Medical School
in partial fulfillment
of the requirements for the degree of
Master of Science

June 1948

APPROVED:

[redacted]

(Professor in Charge of Thesis)

[redacted]

(Chairman, Graduate Council)

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INTRODUCTION

The stages in the oxidative degredation of ascorbic acid have occupied the attention of several groups of investigators over the period of years following the isolation of the compound by Szent-Gyorgyi in 1928[1]. The fact that during these oxidative stages carbon dioxide is given off by a non-oxidative process will be the principal concern of the present study.

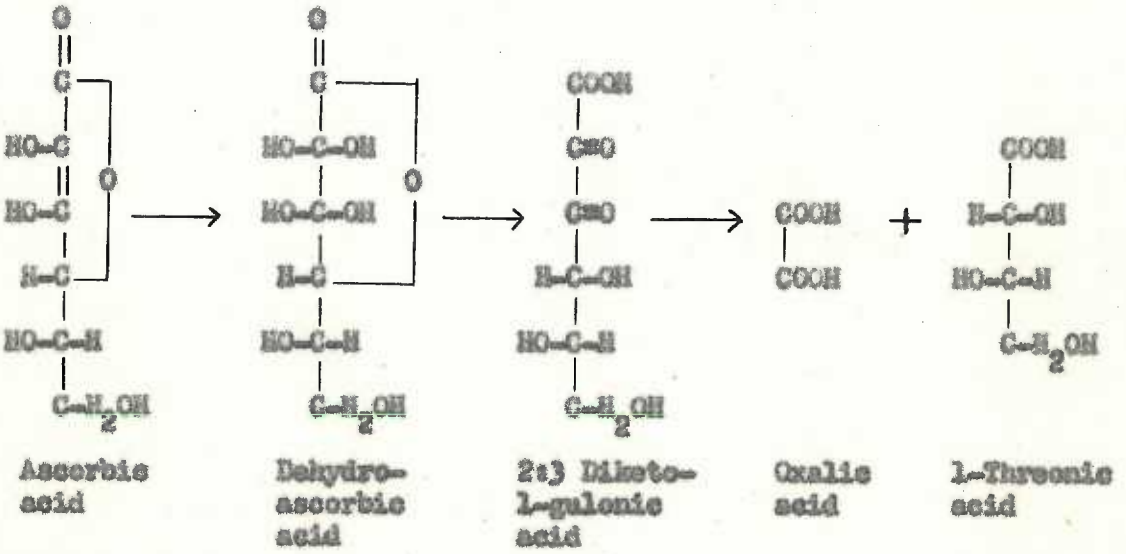
A presentation of the chemistry of ascorbic acid is given to provide a background for the evaluation of the observations made in this study. While working with biological oxidation systems, Szent-Gyorgyi[1] obtained a crystalline substance having the formula $C_6H_8O_6$. Its acidic character, strong reducing properties, and typical color reactions caused it to be classed with the sugar derivatives of the uronic acid group and to be called hexuronic acid. Herbert, Hirst, et al[2], in their classical paper, demonstrated that this compound is not a member of the uronic acid class and sought a more appropriate name. All samples of the compound which had been isolated from plant and animal tissues were found to have anti-scorbutic activity (see references 3,4,5) and, consequently, its name was changed to ascorbic acid by Haworth and Szent-Gyorgyi[6].

The British workers[2] found that the powerful reducing properties of ascorbic acid are evidenced by its reduction of iodine, silver nitrate, copper acetate and potassium permanganate in cold neutral or acid solutions. In addition, alkaline solutions are oxidized by oxygen and Fehling's solution is rapidly reduced in the cold. The presence of at least one enolic carbonyl group was indicated by the ease with which ozasones are formed, by an ultra-violet absorption spectrum similar to many ketonic substances and by color reactions with ferric chloride and sodium

nitroprusside. Quantitative furfural formation on treatment with hydrochloric acid gave evidence that at least five of the six carbon atoms occur in an unbranched chain. Additional investigation[7] showed that a molecule of ascorbic acid contains four hydroxyl groups, two being enolic in character. These observations along with oxidation studies which will be discussed later, led Herbert, et al[2], to state that no free carbonyl group occurs in ascorbic acid and that "the acidic properties are due to the presence of an activated -CHOH group situated next to a carbonyl group, the reactive group being of the type -C(OH):C(OH)-."

A further detailed study of the oxidation of ascorbic acid enabled the British group to complete the determination of the structure of the molecule and initiated an interest by other workers in the stages of oxidation of the substance. The early workers found that the course of oxidation proceeds in two well-defined stages. When an acid solution of iodine is used, two atoms of iodine are required and two molecules of hydriodic acid are released during the oxidation of one molecule of ascorbic acid. Water is necessary for the reaction since alcoholic iodine is without effect. The first oxidation stage occurs without rupture of the molecule since the product can be reduced quantitatively to ascorbic acid by hydrogen sulfide or hydriodic acid. When these workers treated the first oxidation product of ascorbic acid with an alkaline solution of sodium hypoiodite, one atomic equivalent of oxygen was used; and oxalic acid and a trihydroxybutyric acid were detected quantitatively. Methylation and subsequent amide formation gave trimethyl-l-threonamide. The acid was further identified as l-threonic acid on the basis of its conversion into d-tartaric acid on oxidation with nitric acid. By considering the

information obtained in the above experiments, the authors suggested that the molecules involved in the reactions have the following structures and that the following chain of reactions occurs in the alkaline oxidation of ascorbic acid:



It is apparent that the data showed ascorbic acid to be a derivative of l-gulose and its first oxidation product, dehydroascorbic acid, to be converted easily to 2:3-diketo-l-gulonic acid. Dehydroascorbic acid is considered to be a lactone of this diketogulonic acid and to be hydrated. Ascorbic acid is, then, the reduced form of this lactone.

Controversy arose over the validity of this sequence of reactions postulated by the British workers. Much conflicting data has been published because of the fact that the chemistry of dehydroascorbic acid, the primary reversible oxidation product of ascorbic acid, is extremely complex. The compound is formed by oxidation of ascorbic acid with iodine, phenol-indophenol, cupric acetate, ninhydrin, and quinone. The presence of the two free enolic hydroxyl groups in ascorbic acid accounts for the ease of oxidation. Haworth and Hirst[8] claim to have

obtained dehydroascorbic acid, $C_6H_6O_6$, in the solid form and state that it probably exists in aqueous solutions in the hydrated form. The lactone ring, in contrast to that of ascorbic acid, opens readily in water to yield the open chain acid, diketogulonic acid. The lactone form is reduced to ascorbic acid by hydrogen sulfide; the open chain form is not reduced. According to these authors, the opening of the ring, even in acid solutions, gives rise to complex rearrangements because of the fact that the diketo acid can react as a ketose sugar possessing a ring structure. This latter observation is based on the fact that complicated changes occur in the absorption spectrum, rotation, etc. during equilibration of an aqueous solution of diketogulonic acid. In neutral or alkaline solution, complex enolisations may take place.

The comprehensive paper published by Borsook, et al[9], contains pertinent data on the oxidation-reduction potentials of the stages in the oxidation of ascorbic acid. These workers detected three stages of oxidation with several non-oxidative changes occurring between stages. According to their findings, the first stage appears in the pH range from 2 to 4 and results in the oxidation of ascorbic acid to dehydroascorbic acid. In aqueous solutions at pH 4 and at ordinary temperatures, dehydroascorbic acid undergoes a spontaneous, irreversible change to diketogulonic acid. From pH 5.5 to 7.5 diketogulonic acid is oxidized to a postulated unstable intermediary, which breaks down in alkaline media to l-threonic acid and oxalic acid. The third stage takes place rapidly only at a pH greater than 7.0 and possibly is the result of the oxidation of l-threonic acid. It is to be noted that these men simply attempted to correlate their observed physicochemical measurements with the scheme of reactions set forth by Herbert and associates[2] and made no attempt to identify the

products formed. This, however, need not discredit the basic data obtained.

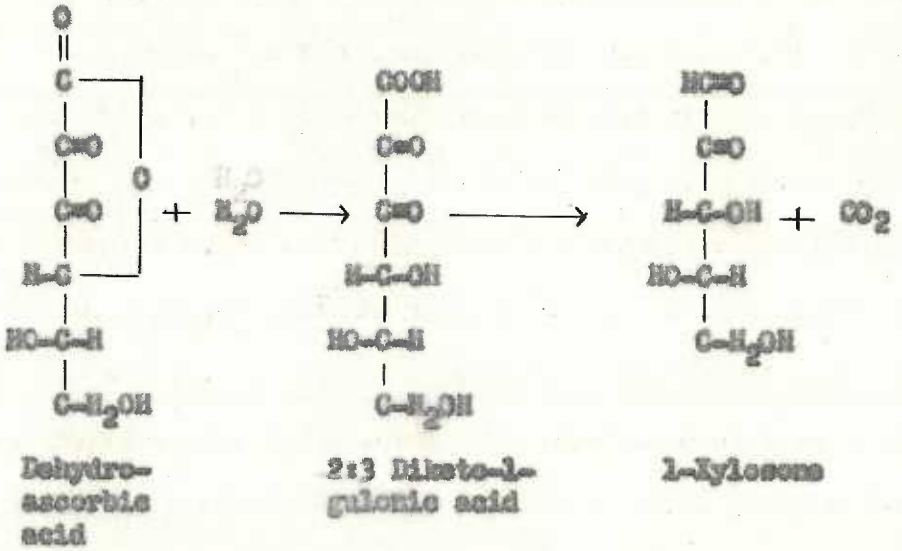
Borsook and his co-workers found that the irreversible change of dehydroascorbic acid presumably to diketogulonic acid was associated with marked changes in the chemical and physiological properties of the substance. Diketogulonic acid is responsible for a negative potential drift observed in electrometric measurements of redox potential. It is a stronger acid than dehydroascorbic acid and a stronger reducing agent than ascorbic acid. It is not reduced to ascorbic acid by hydrogen sulfide or glutathione. It is not antiscorbutic. All of these manifestations of change are dependent on pH; all occur at a pH above 4. All are independent of oxidising agents; therefore, the change is not an oxidation.

The products of oxidation, and the scheme of reactions so far discussed, have been based upon compounds isolated on oxidation of ascorbic acid in alkaline solution. Additional products of ascorbic acid oxidation were obtained by Herbert, Hirst, et al[2] from oxidations carried on in acid solutions. When aqueous solutions of ascorbic acid were treated with potassium permanganate, oxidation occurred almost instantaneously with the utilisation of about one and one-half atoms of oxygen, after which the rate of reaction slowed down. When two atoms of oxygen had been utilised the rate became even slower and carbon dioxide was evolved. Oxalic acid was detected as one product. If 5 N-H_2SO_4 was added to the solution after two atoms of oxygen had been used, the reaction with evolution of carbon dioxide continued at a slow rate until a total of three atoms of oxygen had been used. The resulting solution, made alkaline, was non-reducing. Methylation, followed by amide formation, produced the same trimethyl derivative, trimethyl-l-threonamide, which had been obtained during

alkaline oxidation. No attempt was made to explain the reaction involving the evolution of carbon dioxide upon oxidation of ascorbic acid in acid solutions.

During the second stage of ascorbic acid oxidation, i.e., that following the formation of diketogulonic acid, Lyman and his co-workers[10] also found that the evolution of carbon dioxide could be demonstrated. The evolution was especially noticeable in McIlvaine's citrate-phosphate and in Sorensen's phosphate buffers, but was only slightly noticeable in phthalate-sodium hydroxide buffers.

West and Hinshart[11] in 1942 demonstrated that dehydroascorbic acid, prepared by the oxidation of ascorbic acid with ninhydrin or iodine, loses a molecule of carbon dioxide per molecule of dehydroascorbic acid if the solution is allowed to remain at temperatures varying from 25 to 60° C. until the evolution is completed. The writers suggested that the following series of reactions may take place:



An unsuccessful attempt to isolate and identify l-xylosone was made at that time. It is not unreasonable to assume that the breakdown of

diketogulonic acid passes through the formation of l-xylosone since the synthesis of natural l-ascorbic acid has been accomplished by beginning with this compound[12].

The work reported in this thesis is concerned with the chemistry involved in the evolution of carbon dioxide from oxidized solutions of ascorbic acid. Since methods have become available which permit the determination of all of the postulated intermediates in the production of carbon dioxide from ascorbic acid, these were applied to the problem. A modification of the 2,6-dichlorophenol-indophenol method of Bessey and West[13] was used to determine ascorbic acid and dehydroascorbic acid. The 2,4-dinitrophenylhydrazine method of Roe and Oesterling[14] was used to obtain combined values for dehydroascorbic acid and diketogulonic acid. By appropriate calculations, values for each compound were obtained.

EXPERIMENTAL WORK

A. METHODS

Preparation of Dehydroascorbic Acid.

Dehydroascorbic acid was prepared by a modification of the method of Moll and Wieters[15]. 5.4 g. ascorbic acid in 70 cc. of air-free, ice-cold water are shaken with 3.3 g. quinone in 80 cc. cold ethyl ether for 15 minutes. The aqueous layer is freed of quinone and hydroquinone by shaking three times with 100 cc. portions of cold ethyl acetate and finally with 100 cc. cold ethyl ether. Nitrogen is bubbled through the solution for 30-45 minutes to remove any dissolved ether. A 70-80% yield was obtained as shown by titration with indophenol after reduction to ascorbic acid with H_2S.

Preparation of Quinone.

Quinone was prepared according to directions given by Williams and Brewster[16]. A mixture of 10 g. hydroquinone, 5.5 g. potassium bromate, 100 cc. water, and 5 cc. N-H_2SO_4 is warmed to 60° C. in a 200 cc. flask with stirring. When the black, crystalline quinhydrone, formed initially, is converted to bright yellow quinone, the temperature is raised to 80° C. to dissolve the quinone completely. The solution is then cooled to 0° C., filtered, washed free of potassium bromide with a little ice water, and dried over sodium hydroxide flakes in the refrigerator. The dried crystals are stored in a tightly-stoppered brown bottle, kept in the refrigerator. Precautions are taken to keep the crystalline product cold since quinone has a noticeable vapor pressure at room temperature.

Determination of Diketogulonic Acid and Dehydroascorbic Acid Utilizing the 2,4-Dinitrophenylhydrazine-Ascorbic Acid Method[14]

Reagents:

1. 2,4-dinitrophenylhydrazine: Dissolve 2 g. of reagent in 100 cc. of approximately 9 N-H_2SO_4, filter, and keep in refrigerator.

2. 85% H_2SO_4: To 100 cc. water add 900 cc. concentrated H_2SO_4, sp. gr. 1.84.

3. Metaphosphoric acid-thiourea solution: Prepare a solution containing 5% metaphosphoric acid (HPO_3) and 1% thiourea.

Method:

Dilutions of the solution containing the amount of dehydroascorbic acid in question are made so that the dehydroascorbic acid concentration lies between 0.25γ and 15γ per cc. 4 cc. of the dilution are pipetted accurately into each of three test-tubes. One tube is kept for a blank. To each of the others is added 1 cc. of the 2% 2,4-dinitrophenylhydrazine reagent. The three tubes are held at 37° C. for 3 hours, then cooled in ice water. While the tubes are still in the ice bath, 5 cc. of 85% H_2SO_4 are added drop by drop over a period of not less than 1 minute. 1 cc. of the coupling reagent is then added to the blank, the tubes are removed from the ice water and are kept at room temperature for 30 minutes before readings are taken on a photoelectric colorimeter with a 540 mμ filter.

A standard calibration curve may be prepared with standard solutions of dehydroascorbic acid in concentrations ranging from 0.25γ to 15γ per cc. The dehydroascorbic acid standard is prepared by oxidizing a solution of 25 mg. of ascorbic acid in 25 cc. of 5% metaphosphoric acid with 1 or 2 drops of bromine, shaking until yellow, and aerating until colorless.

Standards of appropriate concentrations are made by diluting aliquot portions with 5% metaphosphoric acid containing 1% thiourea.

This method is based on the formation of osazones with the reagent and the subsequent reaction with sulfuric acid to form a soluble, reddish-colored compound suitable for colorimetric readings. The method was described originally[14,17] for the determination of ascorbic acid and dehydroascorbic acid. Since 2,4-dinitrophenylhydrazine forms the same osazone with ascorbic acid, dehydroascorbic acid, and diketogulonic acid because of the conversion of the first two to diketogulonic acid[18] and since, under the conditions of the method described above, solutions of diketogulonic acid give the same intensity of color as equimolar quantities of ascorbic acid when the latter is oxidized[19], the method was considered suitable, in the present study, for the determination of diketogulonic acid plus dehydroascorbic acid.

A recent paper by Roe and associates[20], dealing with the 2,4-dinitrophenylhydrazine method, shows that only eighty-one per cent of the dehydroascorbic acid present in solution is converted to diketogulonic acid in three hours at 37° C. Only after eight hours at 37° C. does coupling of ascorbic acid with the reagent begin to interfere with the determination of dehydroascorbic acid and diketogulonic acid under the conditions of the method. It will be shown later that this slower coupling of dehydroascorbic acid does not interfere with the indirect determination of diketogulonic acid in our adaptation of the method.

Other interferences would be expected from aldehydes or ketones, which couple with the reagent. These substances do react with 2,4-dinitrophenylhydrazine but do not give the reddish-colored product with sulfuric acid[21]. With the derivatives of pentoses, hexoses, and glucuronic acid,

the brownish color produced on addition of sulfuric acid fades on standing at room temperature for 30 to 45 minutes until there is little or no interference. The fading is believed to be due to the splitting of the dinitrophenylhydrazine compound at the hydrazine linkages with sulfuric acid, and the reformation of the original uncolored substances. Only compounds closely similar in structure to dehydroascorbic acid will give the reddish-colored derivative. Glucose, xylose, fructose, and glucuronic acid have been shown to interfere but this interference can be diluted out[17]. A consideration of interference from products formed under the experimental conditions used in the present work will be given later.

Determination of Ascorbic Acid and Dehydroascorbic Acid by 2,6-Dichlorophenol Indophenol in Xylene

A modification of the method of Bessey and West[13] for determining ascorbic acid with 2,6-dichlorophenol indophenol in xylene was developed for use with the buffered solutions encountered in the course of the present study. In the original procedure a standardized amount of the oxidized form of the dye as the hydrochloride in xylene is shaken with the solution to be analyzed for ascorbic acid and the resulting decrease in color of the dye in xylene is determined by photoelectric colorimetry. The following change was found necessary in the present study: A specified amount of the standardized xylene-dye solution is shaken with alkali solution to convert the dye to the water-soluble, xylene-insoluble salt form. The buffered solution (pH 3.5) containing ascorbic acid is added to the dye solution; a part of the dye is immediately reduced by the ascorbic acid, and the remaining unreduced dye is reconverted to the salt form which is shaken back into the xylene, and the decrease in color is measured in the photoelectric colorimeter.

The following reagents are required:

1. 0.03 N- sodium hydroxide.

2. Citrate buffer, prepared by dissolving 21.0 g. citric acid in 200 cc. of 1.0 N- sodium hydroxide (carbonate-free) and diluting to 250 cc.

3. 3% metaphosphoric acid with citrate buffer, made by using 7 cc. of buffer to 25 cc. of metaphosphoric acid.

4. Xylene.

5. 2,6-dichlorophenol indophenol in xylene. 0.1 g. of dye is extracted with two 25 cc. portions of boiling water, filtered, and diluted to 200 cc. This solution is acidified with hydrochloric acid until red. 200 cc. of xylene are shaken with the solution. The xylene-dye layer is washed several times with 200 cc. portions of 0.03 N-HCl, to prevent fading of the dye by the acid in the ascorbic acid solutions to be analyzed. The xylene-dye layer is dried with anhydrous sodium sulfate and filtered. The solution is then diluted with portions of xylene to a reading of 150-155 on the Klett-Summerson photoelectric colorimeter with a 540 mμ filter.

The procedure for the analysis of ascorbic acid solutions is as follows: A blank is prepared by adding 1 cc. 0.03 N-NaOH to 10 cc. xylene-dye solution in a glass-stoppered graduate followed by shaking about one-half minute until the color is removed from the xylene. 1 cc. of the 3% metaphosphoric acid-citrate buffer solution is added and the dye is shaken back into the xylene (Time: 30 to 60 sec.). The xylene layer is freed of water by centrifuging. The blank is used to set the colorimeter at 150. Samples of the ascorbic acid solution to be analyzed are diluted with the metaphosphoric acid-citrate buffer solution to a point at which 1 cc. contains 0.01 to 0.06 mg. ascorbic acid. The diluted solution is treated

in the same manner as the blank: 1 cc. is added to the basic solution of the dye in xylene and the unreduced salt form of the dye is shaken back into the xylene layer for colorimetric reading. Readings should be made within 30 minutes after the reduction of the dye. It is important that the time of contact of the buffered ascorbic acid solution with the dye and the period of shaking be kept constant.

A standard curve was constructed by treating aliquots of a standard ascorbic acid-metaphosphoric acid-buffer solution in the manner described above. Reproducible curves were obtained.

Dehydroascorbic acid may be determined by the same method after reduction with hydrogen sulfide. The sample containing ascorbic acid and dehydroascorbic acid is diluted with the buffered metaphosphoric acid solution so that one cc. of the dilution contains 0.01 to 0.06 mg. free ascorbic acid. H_2S is bubbled through a portion of the diluted solution for 15 minutes. The H_2S-saturated solution is allowed to stand for two hours, after which time nitrogen is bubbled through for 30-45 minutes to remove the H_2S. This reduced solution, containing total ascorbic acid (dehydroascorbic acid reduced to ascorbic acid plus the original amount of free ascorbic acid) is diluted to contain 0.01 to 0.06 mg. ascorbic acid per cc. and the amount of the compound is then determined as described above. The value obtained for free ascorbic acid subtracted from the value obtained for total ascorbic acid gives the amount of dehydroascorbic acid present in the solution.

A blank was treated with hydrogen sulfide in the above manner. No interference with the xylene-dye reagent occurred from the residual sulfide unremoved by bubbling nitrogen through the solution.

B. PROCEDURES

Solutions of dehydroascorbic acid were prepared as described under "Methods." A few cc. were reserved for initial determinations of ascorbic acid, dehydroascorbic acid and diketogulonic acid; the remaining portion of the solution was divided into two parts, one designated "DHA fraction," to be used to determine the change in concentration of the three substances during the period of the experiment and the other, designated "CO_2 fraction," to be used to determine the amount of carbon dioxide evolved during the same period of time.

To determine the carbon dioxide evolved, a flask containing the CO_2 fraction and a similar flask containing water were connected in a constant-temperature water bath. Dry nitrogen from a cylinder was run first through an absorption tube containing ascarite to remove any CO_2 in the gas, and then into the flask containing only water. The inert gas was then bubbled through the CO_2 fraction at the rate of one bubble per second, then through concentrated sulfuric acid, drierite, and a weighed tube containing a layer of ascarite followed by a layer of drierite. The evolved CO_2 was absorbed by the ascarite in the weighed tube. The difference in the weight of the tube before and after the experiment indicated the weight of CO_2 evolved. The accuracy of the method was checked by acidifying a known amount of carbonate in the reaction flask. The theoretical yield of CO_2 was obtained.

In order that experimental conditions might be the same for the two fractions of dehydroascorbic acid, the flask containing the DHA fraction and, again, a similar flask containing only water were placed in the constant-temperature water bath. Nitrogen was bubbled first through the

flask containing water and then through the flask containing the DHA fraction. This treatment aided also in maintaining a constant volume of the DHA fraction throughout the period of the experimental run.

At the end of the experiment the DHA fraction was allowed to cool to room temperature. Analyses were run on the DHA fraction to determine the amount of ascorbic acid, dehydroascorbic acid, and diketogulonic acid remaining in the solution.

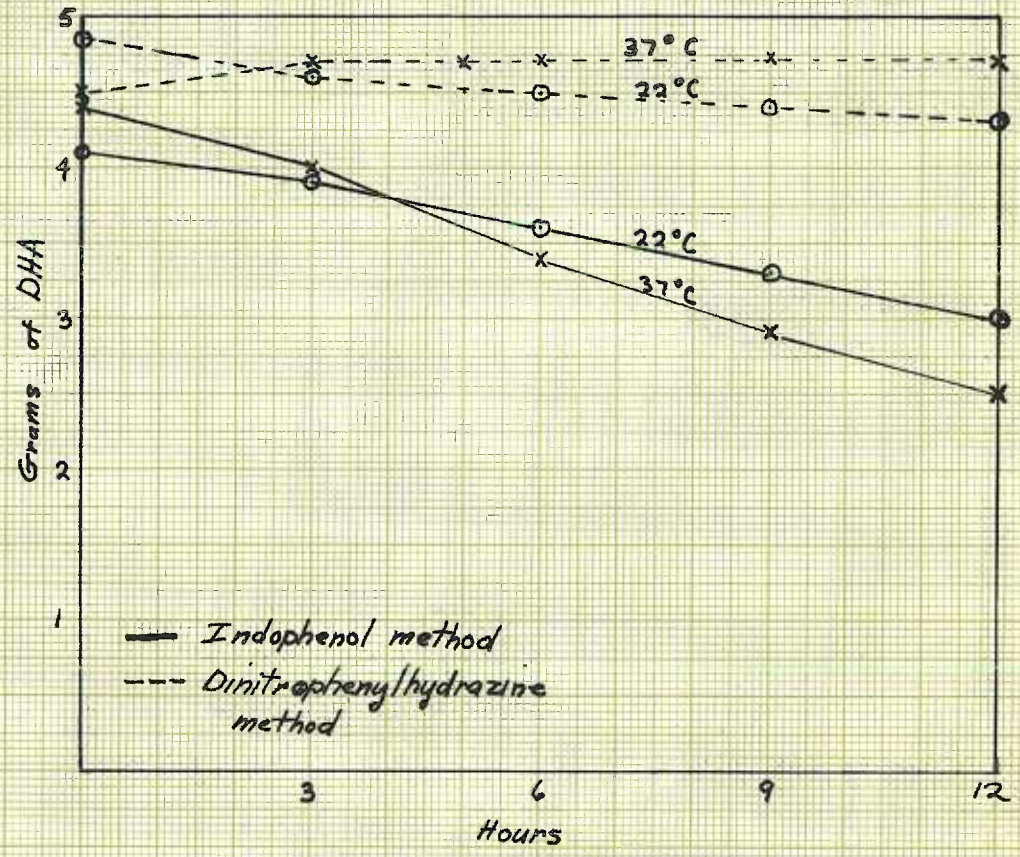
Calculations were made in the following manner: Values for free ascorbic acid were determined by the dichlorophenol indophenol method described. Dehydroascorbic acid was obtained by subtracting the value for free ascorbic acid from the value for the total ascorbic acid determined by the indophenol method after reduction of the solution with hydrogen sulfide. As will be shown later, the amount of diketogulonic acid present at any one time depends upon two different rates of change: the conversion of dehydroascorbic acid to diketogulonic acid and the degradation of diketogulonic acid accompanied by the release of carbon dioxide. It was found unnecessary for our purposes to determine the absolute value of diketogulonic acid. However, since one object of this study is to correlate the change in diketogulonic acid with the evolution of carbon dioxide, the loss of diketogulonic acid is important. Since the method of Roe and Oesterling[14] determines both dehydroascorbic acid and diketogulonic acid, and since dehydroascorbic acid is assumed to be converted to diketogulonic acid before any further changes occur in the molecule, the difference between the values obtained by the dinitrophenylhydrazine method at the beginning and at the end of an experiment was considered to represent the diketogulonic acid lost during that time.

Initially it was necessary to find a temperature at which the decarboxylation reaction takes place at a rate fast enough to show a decided change in the concentrations of the reactants during a three-hour period. In this series of experiments, solutions of dehydroascorbic acid were kept at temperatures ranging from 23° to 60° C. for twelve hours; samples for analysis by the dichlorophenol indophenol and dinitrophenylhydrazine methods were removed at three-hour intervals. The curves in Figure 1, with typical values obtained by the two methods, show the decrease in concentration of the constituents over a twelve-hour period at the temperatures indicated. A temperature of 60° C. was chosen as the one most suited for the work at hand.

C. RESULTS

A series of experiments was run to determine the rate of change for the intermediates in solutions of dehydroascorbic acid. The solutions were maintained at 60° C. for 3, 6, 9, and 12 hour periods. Emphasis should be placed on the fact that the data for each experiment in this group represents values obtained by stopping the reaction at the hour indicated and taking samples for the determinations described under Methods; at no time was an experiment carried on after interruption for sampling. A typical set of values for an experiment is given in Table 1. The method of calculation may be followed from this table.

Figure 1 - Effect of temperature on rate of change of constituents in evolution of carbon dioxide from dehydroascorbic acid



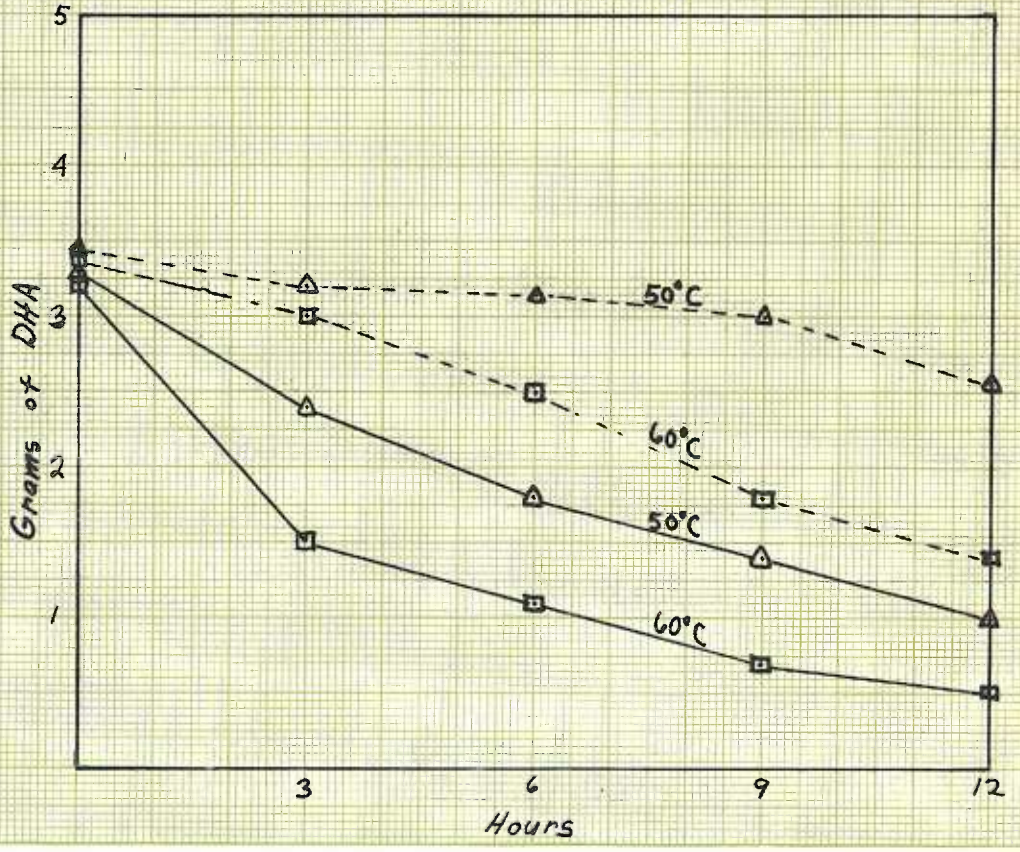


TABLE 1

	Millimols	
	0 hour	6 hours
(1) Free ascorbic acid *	3.25	3.10
(2) Total ascorbic acid *	16.88	6.34
(3) DHA plus DKG **	14.48	9.90
(4) DHA, (2)- (1)	13.63	3.24
(5) DKG, (3)- (4)	0.85	6.66
(6) CO_2 evolved		4.31
(7) DKG lost, (3) at 0 hrs. - (3) at 6 hrs.		4.58
(8) DHA lost, (4) at 0 hrs. - (4) at 6 hrs.		10.39
(9) % error, [(6) - (5)] /(5)		6%

* By dicholorophenol indophenol method.

** By dinitrophenylhydrazine method.

Note: DHA = Dehydroascorbic acid

DKG = Diketogulonic acid

After a number of experimnets had been run, an expected correlation was observed between the values for diketogulonic acid lost and for carbon dioxide evolved; approximately one millimol of diketogulonic acid was lost for each millimol of carbon dioxide detected. With this fact established, data was used only from those experiments showing a difference of 10% or less between these two values. The determination of carbon dioxide was considered to be more accurate than that for diketogulonic acid; therefore, the per cent of error was based on deviations from the values obtained for carbon dioxide. After techniques had been perfected, few experiments were run which showed errors greater than 10%.

Table 2 is a compilation of the main data from which the basic conclusions of this work are derived. In the period of time indicated, each value for dehydroascorbic acid, and for diketogulonic acid lost and carbon

dioxide evolved, is compared with the value for dehydroascorbic acid present at the beginning of the experiment; specifically then, the values show the change in concentration of the substances during the experiment, stated as millimols of substance per millimol of dehydroascorbic acid present at 0 hours. Figures 2, 3, 4, and 5 were drawn from this data. Figures 2, 3, and 4 show the dispersion of values around the average for each experimental period, and Figure 5 indicates the relationships of the curves to each other.

TABLE 2

	3 Hours mM/mM DHA at 0 hrs.	6 Hours mM/mM DHA at 0 hrs.	9 Hours mM/mM DHA at 0 hrs.	12 Hours mM/mM DHA at 0 hrs.
DHA lost	0.40	0.75	0.78	0.86
	0.47	0.66		0.85
	0.61	0.68		0.87
		0.71		
Ave.	0.49	0.70	0.78	0.86
DKG lost	0.14	0.34	0.44	0.70
	0.05	0.20		0.66
		0.28		0.60
		0.24		
Ave.	0.10	0.28	0.44	0.65
CO_2 evolved	0.13	0.32	0.45	0.64
	0.07	0.26		0.61
	0.12	0.27		0.60
		0.26		
Ave.	0.11	0.28	0.45	0.62
DHA present	0.60	0.25	0.22	0.14
	0.53	0.34		0.15
	0.39	0.32		0.13
		0.29		
Ave.	0.51	0.30	0.22	0.14
DKG present *	0.39	0.49	0.44	0.21
	0.52	0.42		0.29
		0.51		0.36
		0.31		
Ave.	0.46	0.43	0.44	0.29

* DKG present at 0 hrs., average value = 0.00 mM/mM DHA at 0 hrs.

Figure 2- Loss of Dehydroascorbic Acid

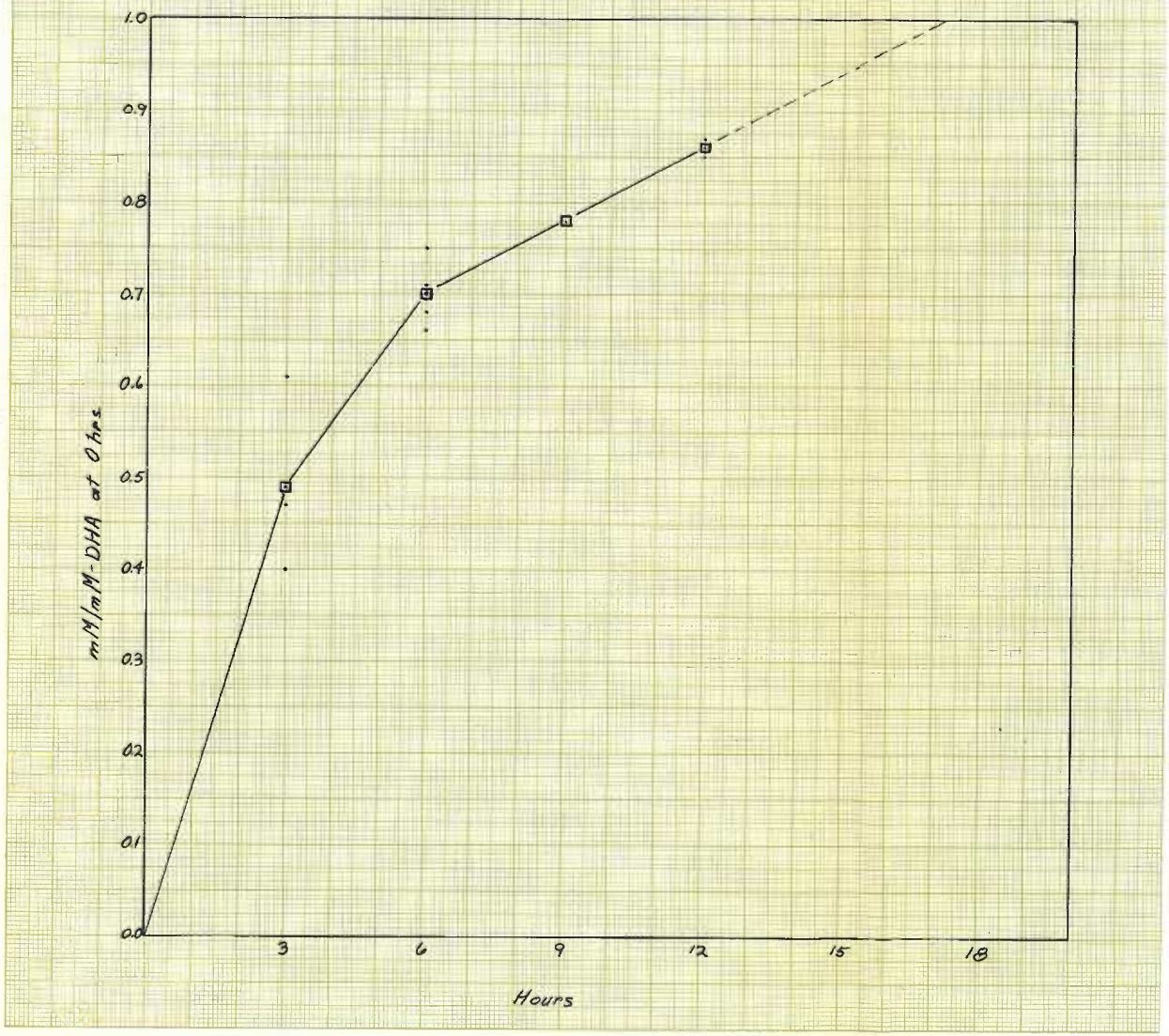


Figure 3 - Disappearance of Diketogulonic Acid

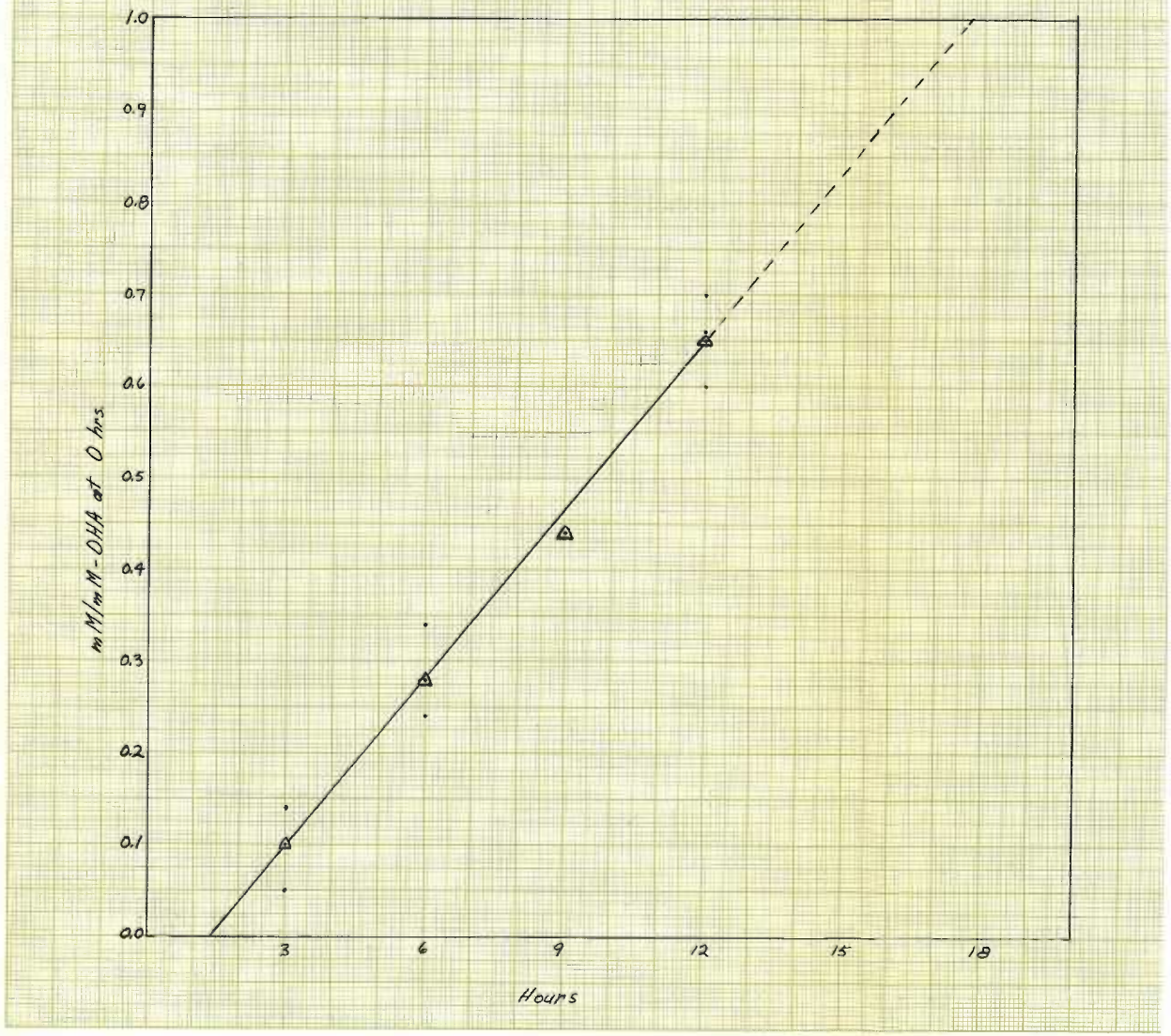


Figure 4 - Evolution of Carbon Dioxide

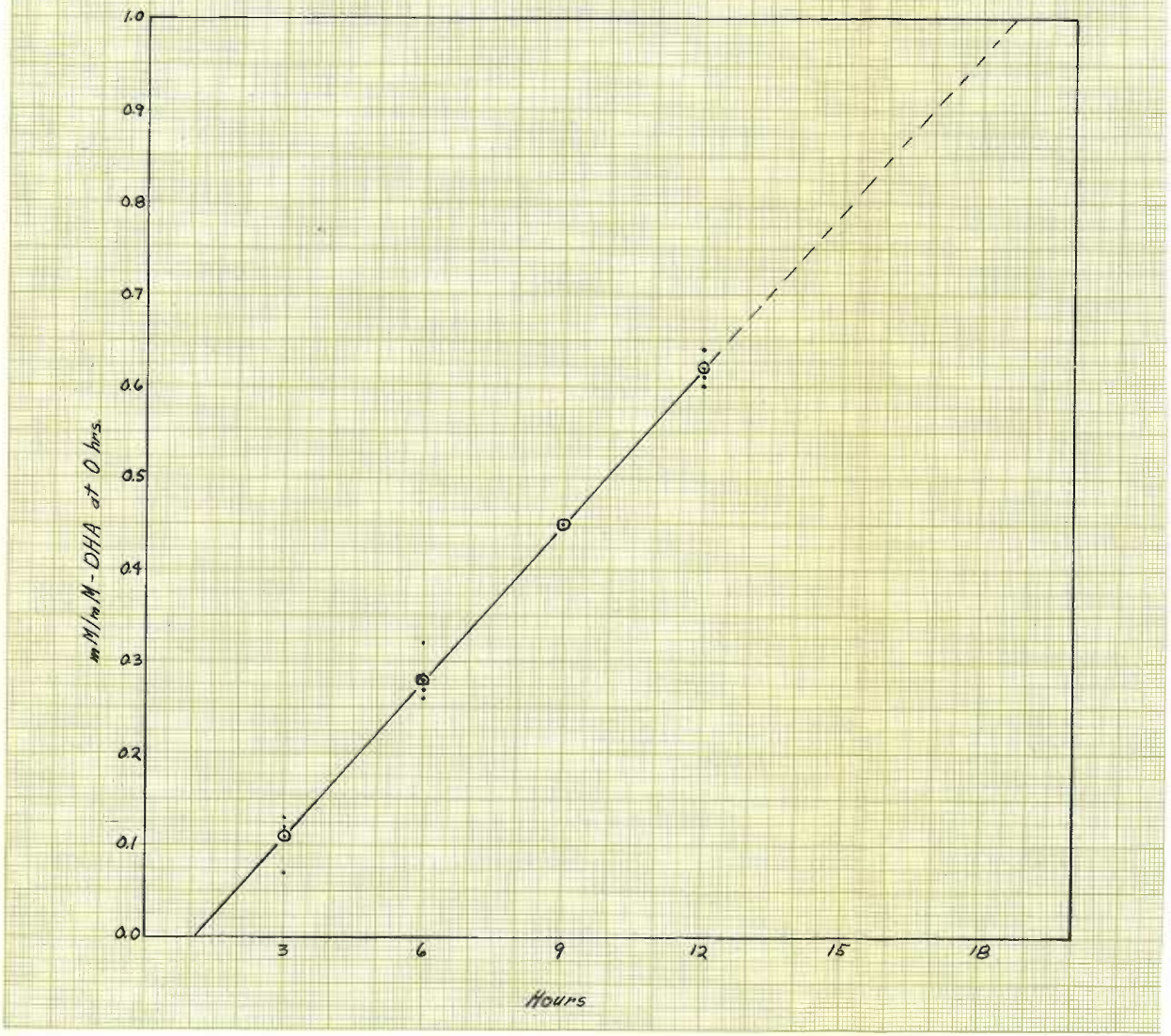
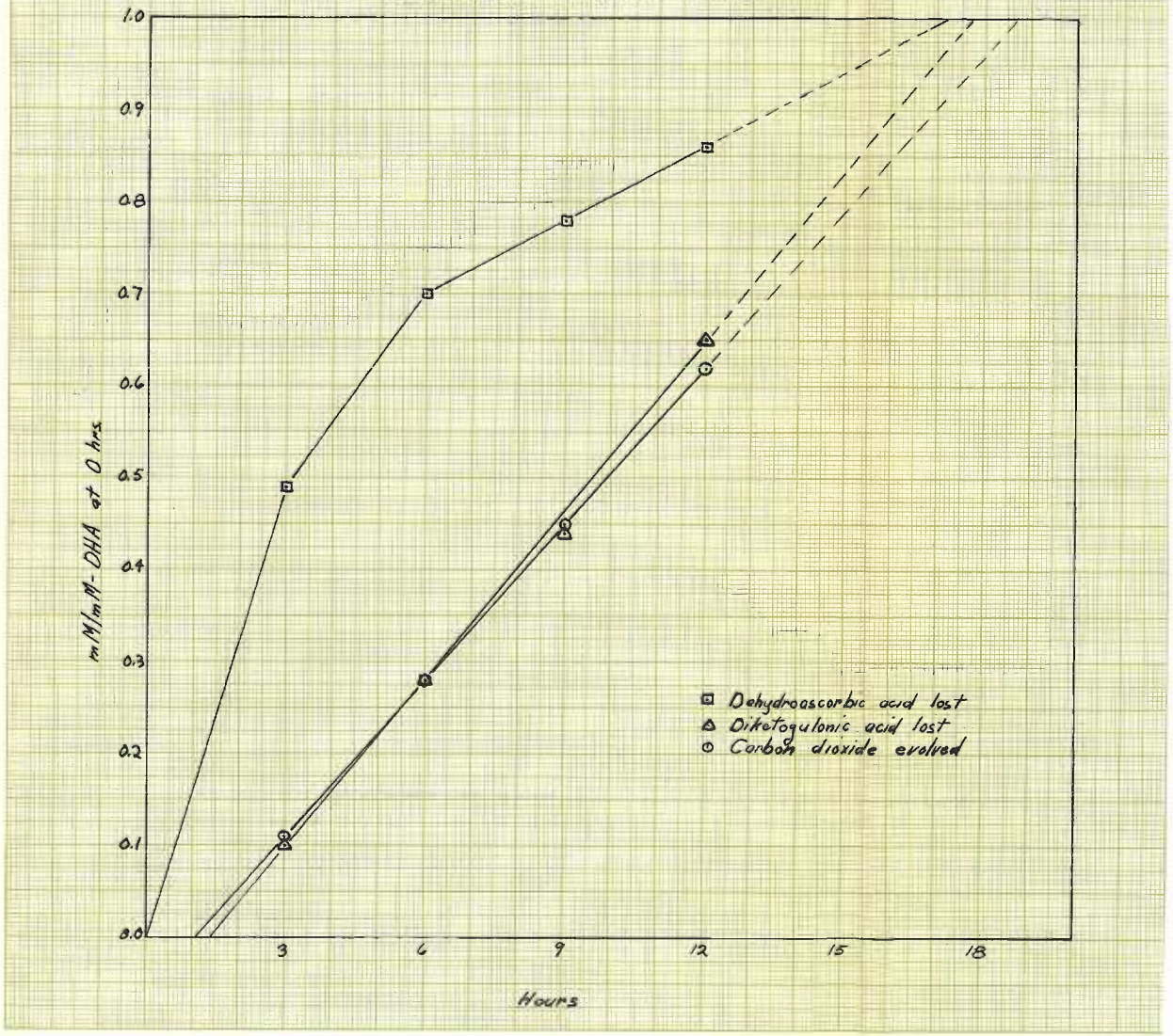


Figure 5 – Relationship of Loss of Dehydroascorbic Acid and Diketogulonic Acid to Evolution of Carbon Dioxide



Examination of Figure 5 shows the rate of loss of diketogulonic acid to be faster, apparently, than the rate of carbon dioxide evolution. Thoughtful consideration of the methods used point to two possible reasons for the discrepancy between the rates. The low values for diketogulonic acid lost during the first 6 hours may be caused by failure to detect all of the diketogulonic acid present by the dinitrophenylhydrazine method. However, error from this source is gradually replaced by a different increasing positive error. The most probable explanation for this increase is a possible interference by a steadily rising concentration of some product, formed during the degredation, capable of coupling with the reagent used. Such a possibility was investigated. A solution of dehydroascorbic acid was maintained at 60° C. for 19 hours, or until carbon dioxide evolution had ceased. The solution was analyzed by the dinitrophenylhydrazine method in the same manner as all other samples. The amount of "apparent" diketogulonic acid was high enough to account for the increasing positive difference between values for loss of diketogulonic acid and evolution of carbon dioxide. The amount of interference from this source at any given time during the evolution of carbon dioxide was impossible to predict. The development of this interference during the twelve-hour period chosen for this work is not sufficient to influence greatly the conclusions to be drawn later.

After six hours a slight amount of interference was also noted in determinations of dehydroascorbic acid by the dichlorophenol indophenol method. The values for free ascorbic acid rose slightly but steadily. In the methods for calculation used, such a rise would result in higher values for the loss of dehydroascorbic acid than would theoretically be expected. When the curves in Figure 5 are extended beyond the twelve-hour

experimental period to the time at which evolution of carbon dioxide ceases, the amount of error occurring from both methods of determination is shown graphically. The error is positive in both cases and, therefore, is in the right direction. Interference by the unknown product is greater in the dinitrophenylhydrazine method than in the indophanol method but is not greater than the 10% considered acceptable for work involving the use of several different techniques.

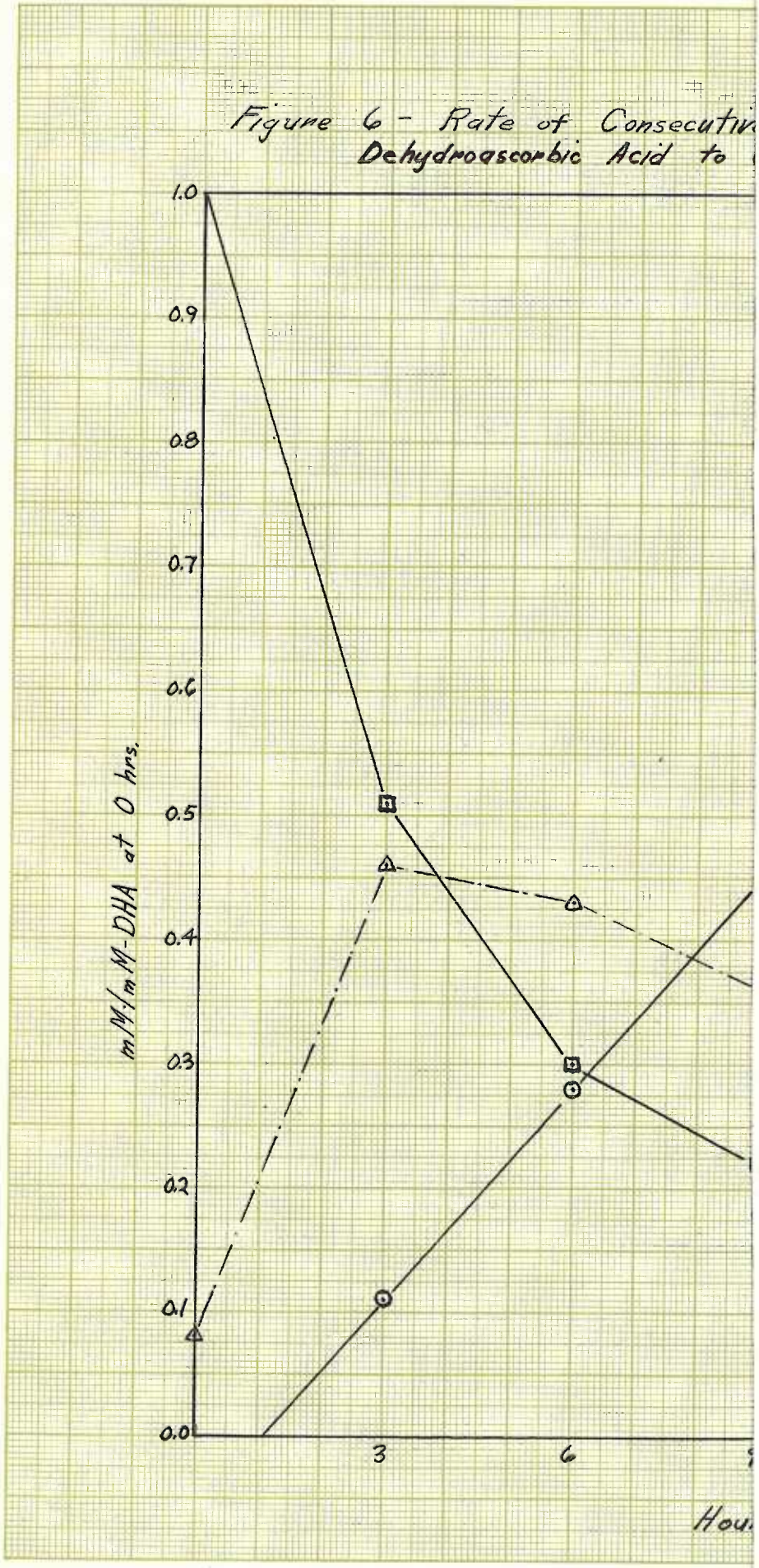
The belief that xylosone is the initial degradation product of diketogulonic acid and that it might be identical with the unknown product causing the interference noted above led to attempts to isolate and identify it. The solutions of dehydroascorbic acid after evolution of carbon dioxide showed the presence of a substance capable of reducing Benedict's solution in the cold. Treatment of these solutions with phenylhydrazine and with bromophenylhydrazine gave good yields of water-insoluble crystalline compounds. However, the product appeared to represent a mixture of substances which could not be separated in a state of purity adequate for identification.

DISCUSSION

The principal findings in this study are embodied in Table 2 and Figure 5. Examination of the data and the curves shows that the greater part of the dehydroascorbic acid is lost during the first six hours and that at the same time the diketogulonic acid, formed from the dehydroascorbic acid, disappears at a linear rate closely approximating the rate of evolution of carbon dioxide. Such a series of consecutive reactions is better represented by the curves in Figure 6. The concentrations of the three reacting substances present at various times is shown graphically. Since the dinitrophenylhydrazine method as used in this work does not determine the absolute amount of dehydroascorbic acid, the values for diketogulonic acid calculated as present at any given time are relative and not absolute. The values for diketogulonic acid present, as determined in Tables 1 and 2, are only indicative of the general change in concentration of this substance over the twelve-hour period of the experiments. Consequently, the curve in Figure 6 for the amount of this compound present, has been dotted in and is used only to show the trend in its change in concentration. From the figure, it will be noted that the concentration of dehydroascorbic acid decreases steadily during the first three hours when the concentration of diketogulonic acid is increasing. At the same time the amount of carbon dioxide evolved increases and, theoretically, equals the initial concentration of dehydroascorbic acid at 19 hours. West and Rinehart[11] showed that at 60° C. 0.50 millimols of carbon dioxide were obtained from 0.58 millimols of dehydroascorbic acid in 24 hours. The figures show that in the consecutive reactions

dehydroascorbic acid ⟶ diketogulonic acid ⟶ carbon dioxide + xylosone (?)

Figure 6 - Rate of Consecutiv
Dehydroascorbic Acid to



there is initially a relatively fast conversion of dehydroascorbic to diketogulonic acid, which is then followed by the slower decarboxylation of diketogulonic acid. The continued conversion of dehydroascorbic to diketogulonic acid coupled with the slower decarboxylation reaction would agree with the shape of the diketogulonic acid curve after three hours.

Further inspection of the data tends to relate the values obtained in this work to a first order reaction. The kinetic equation of a reaction of the first order may be written in the following form:

$$a - x = ae^{-kt}$$

where a = initial concentration of the reacting substance, x = decrease in concentration after lapse of time t, and k = velocity constant. Such a form expresses the fact that the quantity of the reacting material remaining falls off exponentially. On preliminary examination, the curve for the amount of dehydroascorbic acid present appears to follow this form of equation.

For any first order reaction the time taken for a definite fraction of the reacting material to decompose is independent of the initial concentration. In our work, the initial concentration of dehydroascorbic acid ranged from 2 to 4 grams, yet the curves for dehydroascorbic acid present and for carbon dioxide produced imply a rate of reaction independent from these original concentrations. In addition, the values for the evolution of carbon dioxide fit very closely a straight line and also satisfy the above test of a first order reaction. These facts indicate that the consecutive reactions in the evolution of carbon dioxide from dehydroascorbic acid solutions are all of the first order.

Rigid proof of a reaction of this order can be obtained only from substituting experimental values in the equation:

$$k = \frac{1}{t} \ln \frac{a}{a - x}$$

The following table shows the results of applying such a test to our data for the change in concentration of dehydroascorbic acid.

TABLE 3

(a - x)	t	k
0.51 ml	3 hrs.	0.224
0.30	6	0.201
0.22	9	0.168
0.14	12	0.164

Since a constant value for k is not obtained, our data would tend to cast doubt on the evidence obtained by Penney and Zilva[18] that the conversion of dehydroascorbic acid to diketogulonic acid follows a first order reaction. They showed, however, that the rate of reaction is slowest at pH 4 and is accelerated at pH above and below 4. Because the pH of the solutions used in our work changed from 2 to 4, the greater differences in k values for the first six hours may be due to a lower pH, and the better agreement of the k values in the last six hours may be due to a more stable pH.

The studies on the evolution of carbon dioxide clarify certain points concerning the chemistry of dehydroascorbic acid. That evolution of carbon dioxide in the reaction is due to decarboxylation of diketogulonic acid is shown by the curves in figure 5. The millimols of carbon dioxide evolved

correspond to the millimols of diketogulonic acid lost within less than 10 per cent at 0 to 12 hours.

The importance, if any, of this degredation of dehydroascorbic acid to the physiological destruction of ascorbic acid must await further work. Preliminary experiments in which dehydroascorbic acid solutions were maintained at pH 7.0 with phosphate buffers, show that evolution of carbon dioxide occurs. The correlation of the rate of evolution with the disappearance of dehydroascorbic acid and diketogulonic acid at this pH is still to be determined.

The scheme of reactions for the degredation of ascorbic acid as set forth by West and Rinehart[11] has been carried further toward confirmation by this work. Failure to establish the presence of xylosone has been due to the complex nature of the compounds remaining after decarboxylation has occurred.

SUMMARY

The spontaneous evolution of carbon dioxide from solutions of dehydroascorbic acid involves the sequence of consecutive reactions:

dehydroascorbic acid ⟶ diketogulonic acid ⟶ carbon dioxide + l-xylosone (?).

The rate of carbon dioxide evolution parallels the rate of diketogulonic acid destruction. One mole of diketogulonic acid yields one mole of carbon dioxide.

All of the reactions occurring in the degredation of dehydroascorbic acid are believed to be of the first order.

A modification of the ascorbic acid-indophenol method of Bessey and Wood[13] is given for the determination of ascorbic acid in strongly buffered solutions.

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