THE REACTION OF FORMALDEHYDE
WITH ASCORBIC ACID
AND
OTHER ENEDIOL COMPOUNDS

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
Doctor of Philosophy

June 1942

APPROVED:

[redacted]

(Professor in Charge of Thesis)

[redacted]

(For the Committee)

May 16, 1942

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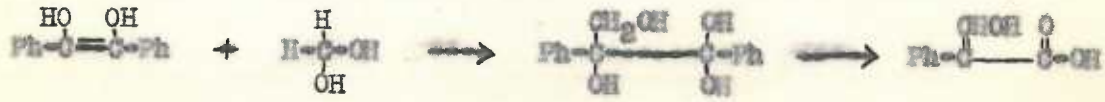
INTRODUCTION

The work embodied in this thesis is an outgrowth of the observations by West and May [1] that formaldehyde reacts with ascorbic acid to produce a syrupy sugar-like product. It was also observed in this laboratory that formaldehyde abolishes the capacity of ascorbic acid to reduce 2,6 dichlorophenol indophenol and iodine [2].

Schmalfuss [3] showed that the condensation of formaldehyde to sugar-like products under the influence of alkalies is markedly catalyzed by the presence of small amounts of reducing sugars. Kusin [4] in a series of papers has shown that, in general, substances possessing the enediol group, $-\overset{HO}{C}=\overset{OH}{C}-$, or which are capable of forming it, catalyze the condensation of formaldehyde to sugars in the presence of alkalies. He demonstrated [5] that ascorbic and isoascorbic acid, both of which contain the enediol group, are active catalysts for this reaction. In addition, he reported that formaldehyde blocks the reducing power of isoascorbic acid, and assumed that an addition product was formed between formaldehyde and the acid.

Kusin succeeded in isolating an addition product from the reaction mixture of formaldehyde and benzoin in alkaline solution. He showed this compound to be hydroxy-methylene benzoin and considered it to be formed as the result of reaction between the enediol form of benzoin and formaldehyde according to the equation:



The purpose of the present research was to study the reaction of formaldehyde and ascorbic acid in detail and to identify the reaction

products insofar as possible. It will be noted that nearly every well-established analytical method in carbohydrate chemistry has been applied to this problem. Only two crystalline compounds, the sine qua non of exact structural analysis, have been obtained. Considerable information has been derived from an intensive and extensive study of the reaction mixture however. This work has been supplemented by observations on the reaction of formaldehyde with other compounds such as reductone, iso-ascorbic acid, glucoascorbic acid, hydroxy tetronic acid, tetronic acid, and glycerophenylose enediol diacetate.

The importance of the research problem is twofold. In the first place, the organic chemistry of ascorbic acid is not well understood. Most of the work on this subject has been done in Europe and has diminished greatly in the last five years. As a compound ascorbic acid is outstanding in that it is a sugar derivative containing a relatively stable enediol group. For forty years the presence of the enediol group in sugars has been the subject of an enormous amount of research and of some controversy. The intermediate formation of the enediol group has been postulated in a great variety of sugar reactions and transformations. The difficulty in studying this group is due to its extreme lability. Ascorbic acid, however, is a compound containing the enediol group in a more stabilized condition and can be studied more conveniently. American workers have published numerous papers on aromatic enediol compounds during the last decade. One of these compounds was synthesized and studied during the course of this research.

A second reason why such an investigation may be of importance is that since the time of Baeyer it has been postulated that the polymerization

of formaldehyde plays a part in photosynthesis. Since ascorbic acid catalyzes the polymerization of formaldehyde and is also present in green leaves where photosynthesis occurs, it has been suggested that ascorbic acid may play a role in photosynthesis. At the present time, however, workers in the field of photosynthesis are not agreed as to whether free formaldehyde molecules, as such, are concerned in the synthesis of carbohydrates by plants. It is certainly true that formaldehyde represents the same state of oxidation as is found in the sugars, and it is difficult to picture the conversion of carbon dioxide to sugar chains without an over-all reduction process equivalent to the production of formaldehyde.

EXPERIMENTAL

Determination of the pK Values of Ascorbic Acid-Formaldehyde Mixtures

A 0.1000 M. solution of ascorbic acid* was titrated in 1 ml. steps with 0.1000 N. NaOH. All water used in these determinations was distilled and deaerated with pure nitrogen. A Beckmann pH meter (glass electrode) was used for the pH determinations.

NaOH ml.	Ascorbic acid ml.	pH 23°C.	
0	-	2.65	
1	9	3.32	
2	8	3.71	
3	7	4.05	
4	6	4.46	
5	5	6.30	
6	4	11.17	
7	3	11.66	
2.5	5	4.05	(pK)

Another similar experiment was made using a solution of 0.1000 M. ascorbic acid in 4 per cent formaldehyde. The 4 per cent formaldehyde used had a pH of 5.95 and a negligible buffer capacity.

NaOH ml.	Ascorbic acid-Formaldehyde ml.	pH 27°C.	
0	-	3.89	
1	9	5.35	
2	8	5.79	
3	7	6.10	
4	6	6.48	
5	5	7.80	
6	4	10.62	
7	3	11.30	
2.5	5	6.21	(pK)

*Pure crystalline Vitamin C made by Hoffmann-La Roche, or supplied by the Mallinckrodt Chemical Works was used in all of the experimental work.

Further data were obtained using 0.1000 M. ascorbic acid in 37% formaldehyde. The 37% formaldehyde used had a pH of 5.69 and a negligible buffer capacity.

NaOH ml.	Ascorbic Acid-Formaldehyde ml.	pH 25°C
0	-	5.11
1	9	6.53
2	8	6.92
3	7	7.19
4	6	7.47
5	5	8.40
6	4	10.02
7	3	10.30
2.5	5	7.23 (pK)

Summary of the effect of formaldehyde on the pK value of ascorbic acid:

Ascorbic acid in water	4.08
Ascorbic acid in 4% formaldehyde	6.21
Ascorbic acid in 37% formaldehyde	7.23

Determination of the pK Values for Reductone-Formaldehyde Mixtures

The experimental details were exactly the same as those in the work with ascorbic acid.

Reductone in water	5.05
Reductone in 4% formaldehyde	6.10
Reductone in 37% formaldehyde	7.70

Change of pH during the Reaction between Ascorbic Acid and Formaldehyde

0.25 gms. ascorbic acid were dissolved in 25 ml. distilled water. 25 ml. of 37% formaldehyde were added. The mixture was allowed to react in an atmosphere of nitrogen at 30°C.

Time in hours	pH 25°C.
0.0	4.40
0.5	4.30
1.0	4.32
1.5	4.30
2.0	4.30
3.5	4.26
7.5	4.35
18.0	4.63

2.0 gms. ascorbic acid were dissolved in 5.0 ml. of 37% formaldehyde having a pH of 5.95. The mixture was allowed to react in a nitrogen atmosphere at 60°C.

Time in hours	pH 25°C.
0	3.68
16	3.35
48	3.02
60	3.00

0.5 gms. ascorbic acid were dissolved in 10.0 ml. of 37% formaldehyde having a pH of 7.3. The mixture was allowed to react at 50°C. while nitrogen was bubbled through.

Time in days	pH 25°C.
0	4.70
5	3.75
10	3.50
15	3.40

Changes in Optical Rotation during the Reaction of Ascorbic Acid and Formaldehyde

1.00 gms. ascorbic acid was dissolved in about 30 ml. of 19% formaldehyde, and made up to exactly 50 ml. with 19% formaldehyde.

Readings were made in a polariscope using a 2 dm. tube. An electric sodium vapor lamp was used as a light source. The solution was allowed to remain in the polariscope tube at room temperature (21°C.) throughout the experiment.

Day	Time	$[\alpha]_D^{21°}$
0	11:00 AM	+ 33.4°
	3:00 PM	+ 31.5°
1	9:15 AM	+ 30.7°
	8:00 PM	+ 47.5°
2	10:45 AM	+ 45.0°
5	1:30 PM	+ 25.0°
10	10:30 AM	0.0°
13	10:30 AM	- 11.2°
17	10:30 AM	- 25.0°
19	10:30 AM	- 22.5°*

Control compounds, in the same concentration as the ascorbic acid in the above experiment, had the following specific rotations:

Ascorbic acid in water $[\alpha]_D^{24°}$ + 21.1°

Purified reaction product of ascorbic acid and formaldehyde in water $[\alpha]_D^{21°}$ - 16° to - 18°

Purified reaction product of ascorbic acid and formaldehyde in 19% formaldehyde $[\alpha]_D^{21°}$ - 21.7° to - 22.9°

An experiment identical to the one above was set up. The reaction was carried out at 60°C. instead of 21°C.

Time in hours	$[\alpha]_D^{21°}$
0	+ 33.4°
5	+ 2.9°
10	- 22.2°

* This value was obtained after releasing the CO_2 pressure which had developed during the reaction.

Production of Carbon Dioxide during the Reaction between Ascorbic Acid and Formaldehyde

Mixtures of ascorbic acid and formaldehyde were allowed to react in a flask immersed in a water bath. Pure nitrogen was bubbled through the mixture. The gas was led from the flask through an absorption spiral immersed in 0.2 N. $Ba(OH)_2$. At the end of the experiment the $Ba(OH)_2$ was titrated with 0.1 N. HCl to a phenolphthalein endpoint.

0.250 gms. ascorbic acid were dissolved in 25 ml. CO_2 free water and 25 ml. of 37% formaldehyde were added to the solution. This mixture was allowed to react in the above described apparatus for five hours at 60°C.

CO_2 found ------0.0622 gms.

Theoretical -----0.0622 gms. assuming one mol of CO_2 per mol ascorbic acid.

Control runs on ascorbic acid in water and those on formaldehyde in the same concentrations as above, and under the same conditions of time and temperature, yielded no CO_2.

Another experiment was carried out using the same concentrations, but at 39°C., for 23 hours.

CO_2 found ------0.0255 gms. per cent theoretical = 41

The experiment was continued for 24 hours.

CO_2 found ------0.0180 gms. per cent theoretical = 29

For the total 47 hour period:

CO_2 found -----0.0435 gms. per cent theoretical = 70

The effect of varying the concentration of formaldehyde was also studied. In each experiment 0.250 gms. ascorbic acid were dissolved in 25 ml. water and 37% formaldehyde was added to obtain the desired concentration.

Formaldehyde per cent	Time hours	C.°	CO_2 gms.	Theoretical per cent
2	24	50	0.0271	40
4	22	60	0.0442	70
6	22	60	0.0493	80
9	6	60	0.0343	55
11	6	60	0.0354	56
14	5	60	0.0453	70
16.5	5	60	0.0574	92
19	5	60	0.0591	93

Finally, several experiments were set up using 0.250 gms. ascorbic acid in 50 ml. of 19% formaldehyde. These mixtures were allowed to react at 50°C. for periods as long as seven days. In no case was there produced more than one mol of CO_2 per mol of ascorbic acid.

The formation of gas in polariscope tubes containing mixtures of ascorbic acid and formaldehyde has been noted previously. The contents of these tubes, when allowed to stand for about two weeks at room temperature, evolved CO_2 vigorously when opened. The formation of gas may be observed readily when such mixtures are allowed to react in fermentation tubes.

Determination of the Minimum Amount of Formaldehyde Necessary to Block the Indophenol Reducing Power of Ascorbic Acid

Crystalline ascorbic acid was added in small amounts to 50 ml. of 37% formaldehyde until the mixture reduced 2,6 dichlorophenol indophenol. Throughout the experiment 30 minutes were allowed for reaction after

the solid portions were dissolved. 22.5 gms. ascorbic acid were added before the mixture reduced more than 1 ml. of a saturated aqueous solution of 2,6 dichlorophenol indophenol. The ratio of mols of ascorbic acid to mols of formaldehyde was 1 : 4.95.

Determination of the Amount of Formaldehyde Bound by Ascorbic Acid.

Nordlander's modification of Cambrier's method for the estimation of formaldehyde was used [6]. In general, the method is as follows: To a 1.00 ml. sample are added 2 drops of bromphenol blue. The solution is neutralized with 0.5 N. HCl, and then a drop of 0.5 N. NaOH is added to bring back the basic color of the indicator. 5 to 15 ml. of 10% hydroxylamine hydrochloride are then added, the mixture shaken and allowed to stand for ten minutes. The HCl liberated is titrated with 0.5 N. NaOH.

Sample	0.5000 N. NaOH ml.
1.00 ml. 37% formaldehyde	26.5
1.00 ml. aliquot of a mixture of 22.5 gms. ascorbic acid in 50 ml. of 37% formaldehyde (total volume 64 ml.) (1.00 ml. mixture contains 0.78 ml. of 37% formaldehyde.)	18.8
as above, after two days at 23°C.	18.8
as above, after ten days at 23°C.	18.8
as above, after four days at 50°C.	18.8

If no formaldehyde were bound, 1.00 ml. of the above ascorbic acid-formaldehyde mixture would have a titration value of 20.6 ml. (0.78 x 26.5). The difference observed was 1.8 ml. (20.6 - 18.8). 1.8 ÷ 20.6 = 0.0875 or 8.75 %. In the mixture, the ratio of mols of ascorbic acid to mols of formaldehyde is 1 : 4.95. 0.43 mol (0.0875 x 4.95) of formaldehyde was bound by 1 mol of ascorbic acid.

Formaldehyde may be determined by the following method also [7]: A sample containing formaldehyde is allowed to react with excess 5% $(NH_4)_2SO_4$ for exactly 15 minutes. Three drops of rosolic acid in ethanol are added. The mixture is then made basic with 0.5 N. NaOH. The excess base is titrated with 0.5 N. HCl. This method yielded the same titration values for ascorbic acid-formaldehyde mixtures as for pure formaldehyde controls.

In another method, formaldehyde may be determined by utilizing the reaction between formaldehyde and KCN [8]. To 10 ml. of 1% KCN is added 1 ml. concentrated HNO_3 and 1 ml. of the sample containing formaldehyde. Then 10 ml of 0.2 N. $AgNO_3$ are added. The excess CN^- precipitates as AgCN and is filtered off. The excess $AgNO_3$ is determined by titrating with thiocyanate. Purified samples of the ascorbic acid-formaldehyde reaction product were found to bind KCN and thus could not be distinguished from formaldehyde.

Formaldehyde is oxidized quantitatively to formic acid by alkaline H_2O_2 [9]. 50 ml. of N. NaOH and 50 ml. of 3% H_2O_2 are mixed. 5 ml. of the sample containing formaldehyde are added. The mixture is heated on the steam bath for 5-15 minutes. When cool, a few drops of litmus are added, and the mixture titrated with N. HCl. As a check, the formic acid may be determined as follows: 10 ml. of 50% sodium acetate, 2 ml. of 10% HCl, and 25 ml. of a mercury reagent (containing 100 gms. $HgCl_2$ and 150 gms. NaCl per liter) are added and the mixture heated on the steam bath for two hours. The precipitated HgCl, equivalent to the weight of formaldehyde in the sample, is filtered, dried, and weighed. The reaction product of

ascorbic acid and formaldehyde, when oxidized as above, also yielded formic acid.

In the presence of other aldehydes, formaldehyde may be determined by using Schiff's reagent [10]. 5 ml. of the sample containing formaldehyde, 5 ml. Schiff's reagent, and 1.2 ml. of 75% H_2SO_4 are mixed and allowed to stand in a stoppered test tube for two hours. The color due to aldehydes other than formaldehyde fades completely in this time. This method is useful only for solutions containing less than 0.03% formaldehyde. The color developed by the sample is compared in a colorimeter with that developed by a known concentration of formaldehyde. 37% formaldehyde was diluted 1 : 1000. The color developed by this was compared with that developed by a 1 : 1000 dilution of 2.2 gms. of ascorbic acid in 5.0 ml. of 37% formaldehyde. No difference could be detected.

In another set of experiments, formaldehyde was allowed to react with an excess of ascorbic acid in an atmosphere of nitrogen at 50°C. for three days. The mixtures were as follows:

Mixture	Ascorbic acid gms.	0.037% formaldehyde ml.
1	0.20	5.0
2	0.40	5.0
3	0.50	5.0

When tested with Schiff's reagent after three days, mixtures 1 and 2 yielded just a trace of color; mixture 3 was completely negative.

Further, three identical mixtures were made up, each containing 0.10 gms. ascorbic acid and 5.0 ml. of 0.037% formaldehyde. They were allowed to react in an atmosphere of nitrogen at 50°C. On the second, third, and seventh days, they all gave a strong color with Schiff's reagent.

Reaction between Crystalline Ascorbic Acid and the Vapor from Formaldehyde Solutions

A desiccator was fitted so that nitrogen could be passed through it. The exit gas was led through an absorbing spiral immersed in 0.2 N. $Ba(OH)_2$. An evaporating dish containing a layer of crystalline ascorbic acid and another containing 37% formaldehyde were placed in the desiccator. The desiccator was flushed out with nitrogen and the absorbing spiral was connected. Within a few hours at 25°C. appreciable $BaCO_3$ could be observed in the $Ba(OH)_2$ solution and the ascorbic acid became a thick syrup. Several preparations made in this manner yielded products which had the same optical rotation as those obtained from mixtures of ascorbic acid and formaldehyde solutions.

The Reaction of Ascorbic Acid with Compounds Similar to Formaldehyde

Mixtures of ascorbic acid and formic acid were made as follows:

Solution	Ascorbic acid gms.	Water ml.	Formic Acid 85% ml.
A	0.500	20.0	0.0
B	0.500	19.0	1.0
C	0.500	10.0	10.0
D	0.500	0.0	20.0

Using a calibrated 0.1 ml. pipette, 0.10 ml. samples of the above solutions were titrated with a saturated aqueous solution of 2,6 dichlorophenol indophenol.

Solution	Titration values in ml.		
	0 hr.	18 hr.	18 days
A	46	44.5	37.8
B	47	42.5	28.5
C	46	39.5	7.0
D	46	41.5	4.0

Similar experiments with hexamethylene tetramine, acetaldehyde, and benzaldehyde did not indicate any effect on the indophenol reducing power of ascorbic acid.

In another series of experiments 10% solutions of ascorbic acid in water were mixed with hexamethylene tetramine, with acetaldehyde, with benzaldehyde, and with formic acid. These mixtures were allowed to stand in fermentation tubes at 25°C for several weeks. No production of gas was noted as in the control containing the same concentration of ascorbic acid and formaldehyde equivalent to the other substances.

The Reaction of D-Iso-Ascorbic Acid with Formaldehyde.

The formulas of ascorbic and d-iso-ascorbic acids are:

d-iso-ascorbic acid

l-ascorbic acid

The sample of iso-ascorbic acid used was purchased from Eastman Kodak Co. and was not further purified.

0.25 gms. d-iso-ascorbic acid was dissolved in 25 ml. of 10% formaldehyde and allowed to stand 15 minutes. This mixture reduced 0.2 ml. of a saturated solution of 2,6 dichlorophenol indophenol. The same quantity of d-iso-ascorbic acid in water reduced more than 30 ml. of the indophenol solution.

One gram of d-iso-ascorbic acid was dissolved in 37% formaldehyde and made up to a volume of exactly 25 ml. with 37% formaldehyde. Readings were made in a 2 dm. tube.

Day	Time	$[\alpha]_D^{25°}$
0	2:30 PM	+75.0°
1	7:30 AM	+86.2°
2	3:00 PM	+85.7°
3	4:30 PM	+90.0°
5	10:00 AM	+80.0°
9	10:00 AM	+72.5°
18	2:00 PM	+60.0°
40	5:00 PM	+43.7°

At the same concentration (1 gm. in 25 ml.) d-iso-ascorbic acid in water had a value of $[\alpha]_D^{25°}$ -18.7°. It was noted that gas was formed in the closed polariscope tube during the determination. By the use of the same methods described for ascorbic acid, this gas was shown to be CO_2.

5 gms. of d-iso-ascorbic acid were spread out in an evaporating dish and placed in a desiccator next to another evaporating dish containing 50 ml. of 37% formaldehyde. The desiccator was set in a cupboard at 30°C. for 18 days. The iso-ascorbic acid became a viscous syrup. The syrup was diluted with water and transferred to a round-bottomed flask. The excess formaldehyde was removed by vacuum distillation, followed by re-solution in water and repeated distillation at 30°C. The residual material was dried in vacuo. This product was a light brown, non-crystalline solid which reduced Benedict's sugar reagent when warmed.

The Reaction of D-Gluco-Ascorbic Acid with Formaldehyde.

D-gluco-ascorbic acid has the formula:

The sample used was purchased from Eastman Kodak Co. and was not purified further.

0.25 gms. d-gluco-ascorbic acid were dissolved in 25 ml. of 19% formaldehyde and allowed to stand 15 minutes. This mixture reduced 0.2 ml. of a saturated aqueous solution of 2,6 dichlorophenol indophenol. The same quantity of d-gluco-ascorbic acid in water reduced over 30 ml. of the indophenol solution.

1 gm. of d-gluco-ascorbic acid was dissolved in 37% formaldehyde and made up to a volume of exactly 25 ml. with 37% formaldehyde. Readings were made in a 2 dm. tube.

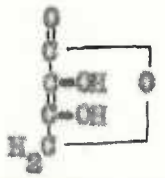
Day	Time	$[\alpha]_D^{25^\circ}$
0	2:30 PM	-21.7°
	3:30 PM	-24.1°
	5:00 PM	-25.7°
1	8:30 AM	-25.7°
2	11:30 AM	-24.4°
6	4:30 PM	-23.4°
9	5:00 PM	-23.7°
16	3:30 PM	-22.5°
37	3:30 PM	-22.5°

At the same concentration (1 gm. in 25 ml.) d-gluco-ascorbic acid in water had a value of $[\alpha]_D^{25^\circ}$ -22.6°.

1 gm. of d-gluco-ascorbic acid was dissolved in 25 ml. of 37% formaldehyde in a small flask immersed in a water bath at 45°C. The mixture was aerated with nitrogen and the gas leaving the flask was led through an absorbing spiral immersed in 0.2 N. $Ba(OH)_2$. After 24 hours but little $BaCO_3$ had formed, after 48 hours $BaCO_3$ was present in quantity. When reaction was complete (no more $BaCO_3$ formed), the mixture was found to reduce hot Benedict's sugar reagent.

Reaction of α-Hydroxy-Tetronic Acid with Formaldehyde

α-hydroxy-tetronic acid has the formula:



This compound was made by the method of Michael and Jung [11]. Since the method required benzoyl ethyl glycolate, this compound was also prepared.

50 gms. Eastman ethyl glycolate were mixed with 100 ml. dry $CHCl_3$ and 100 ml. pure, dry pyridine. 100 ml. benzoyl chloride were added dropwise with stirring over a two-hour period. The temperature of the mixture was kept below 50°C. The mixture was allowed to stand over night. 30 ml. of water were added and the mixture allowed to stand for a half hour. The whole was diluted with ice water and extracted thoroughly with $CHCl_3$. The $CHCl_3$ solution was washed well with normal solutions of H_2SO_4 and $NaHCO_3$ and with water. The $CHCl_3$ solution was dried with anhydrous Na_2SO_4 and the $CHCl_3$ removed at the water pump. The syrup obtained was dissolved in ethanol and poured into a large quantity of water with vigorous stirring. The syrup was dried again and distilled under the vacuum of an oil pump. About 60 gms. of benzoyl ethyl glycolate were obtained, boiling at 98°C at a pressure of 0.1 mm. Hg.

20 gms. benzoyl ethyl glycolate and 50 ml. dry benzene were put in a three-necked 500 ml. flask fitted with a condenser and a mercury-sealed stirrer. Dry nitrogen was bubbled through the solution which was heated to 100°C. in an oil bath. 5 gms. metallic potassium were added

in small quantities. The whole was refluxed with stirring for three hours. When cool, 5 ml. concentrated H_2SO_4 in 45 ml. water were added and the mixture was stirred. The aqueous layer was pipetted off and evaporated in vacuo at 30°C. The solid obtained was extracted with absolute ethyl acetate. The ethyl acetate solution was evaporated to a thick liquid, a small amount of ether was added, and the mixture allowed to stand in an atmosphere of CO_2. Crystallization took place within an hour. The crystals were dried on a porous plate. This material had a melting point of 148°C. (literature 152°C.). As judged by iodine titration, the purity was 98%. The yield was 0.5 gms.

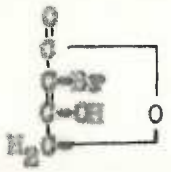
0.0097 gms. of α-hydroxy-tetronic acid reduced 8.05 ml. of 0.0202 N. iodine solution. (Theoretical is 8.19 ml.).

0.0084 gms. of α-hydroxy-tetronic acid were dissolved in 10 ml. of 37% formaldehyde and allowed to stand for a half hour. The mixture, when titrated, reduced 0.6 ml. of 0.0202 N. iodine.

0.1 gms. α-hydroxy-tetronic acid was dissolved in 10 ml. of 37% formaldehyde. The solution was placed in a large test tube so that nitrogen could be bubbled through it. The exit gas was led through an absorbing spiral immersed in 0.2 N. $Ba(OH)_2$. After 24 hours at 25°C. no $BaCO_3$ was present. When the temperature was raised to 35°C., a precipitate of $BaCO_3$ was evident in 24 hours. After reaction was complete (cessation of CO_2 evolution), the mixture still reduced Benedict's reagent readily when warmed.

Reaction of α-Bromo-Tetronic Acid with Formaldehyde

α-bromo-tetronic acid has the formula :



This compound was made by the method of Wolff and Schwabe [12].

200 gms. Eastman ethyl acetoacetate (practical) and 200 ml. dry ether were mixed in a three-necked liter flask equipped with a dropping funnel, condenser, and mercury-sealed stirrer. The flask was immersed in an ice bath. 492 gms. bromine were added to the mixture in the flask with vigorous stirring over a period of two hours. The mixture was allowed to stand over night, washed well with ice water, and dried over $CaCl_2$. This crude preparation of ethyl α,γ -dibromo acetoacetate was then heated in a Claisen flask at 125°C. for three hours under water pump vacuum. The flask was cooled in ice, and the crystals filtered off. The filtrate was heated again in the same way, cooled and filtered; the process being repeated until no more solid material separated. The impure α -bromo-tetronic acid was recrystallized from ethyl acetate. The melting point was 183°C. dec. (literature 185°C. dec.). The yield was 20 gms.

Since α -bromo-tetronic acid liberates iodine from KI, this property was used as a criterion for determining whether formaldehyde reacts with it.

0.1 gm. α-bromo-tetronic acid was dissolved in 10 ml. water. 1.0 ml. of this solution, when mixed with 5 ml. of 0.1 N. HCl and 5 ml. of 10% KI, liberated iodine equivalent to 12.63 ml. of 0.0100 N. $Na_2S_2O_3$. 1.0 ml. of the α-bromo-tetronic acid solution was mixed with 5 ml. of 37% formaldehyde and allowed to stand for a half hour. 5 ml. of 0.1 N. HCl and 5 ml. of 10% KI were then added. The iodine liberated was equivalent to 12.68 ml. of 0.0100 N. $Na_2S_2O_3$.

Reaction of Tetronic acid with Formaldehyde

Tetronic acid has the formula:

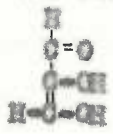
This compound was prepared by the method of Wolff and Schwabe [13].

7.4 gms. of α-bromo-tetronic acid were dissolved in the smallest possible amount of cold 20% Na_2CO_3. The solution, in a small flask, was cooled to 0°C. in an ice bath and CO_2 was bubbled through. 100 gms. of 4% sodium amalgam were added in 10 gm. portions over a period of five hours, and the reaction mixture was allowed to stand at 0°C. over night. The mixture was acidified with 1 : 1 H_2SO_4, filtered, and extracted with ether. When the ether extract was evaporated, about 0.3 gms. of white crystals were obtained, melting at 140°C. The m.p. given in the literature for tetronic acid is 141°C.

0.1068 gms. tetronic acid were dissolved in 10 ml. water. 5 ml. of water were added to 5 ml. of this solution. The mixture reduced 0.6 ml. of 0.02 N. iodine. To the other 5 ml. of the tetronic acid solution were added 5 ml. of 37% formaldehyde and the mixture was allowed to stand for half hour. When titrated it reduced 0.05 ml. of 0.02 N. iodine.

Reaction of Reductone with Formaldehyde

Reductone has the formula:



This compound had been prepared previously in this laboratory by the method of Euler and Martius [14], and was recrystallized from

water until it had the melting point, 200°-220°C., recorded in the literature.

The data showing the effect of formaldehyde on the pK of reductone solutions were presented in the first part of the experimental section of this thesis.

5 gms. reductone were dissolved in 25 ml. of 37% formaldehyde. After 15 minutes this mixture no longer reduced 2,6 dichlorophenol indophenol. The mixture was allowed to stand at 30°C. for two weeks and then the excess formaldehyde was removed by repeated vacuum distillation of the aqueous solution at 50°C. until the distillate no longer gave a precipitate with dimedon. The reaction product was dried in vacuo and placed in a desiccator over Drierite. After several weeks this material became a hard yellow solid which appeared to crystallize. When small fragments were viewed under a polarizing microscope, however, no birefringence could be observed, nor did they possess a sharp melting point. The material reduced Benedict's reagent readily when warmed; was soluble in methanol, methyl cellosolve, and pyridine, but was insoluble in ether.

4.6 gms. of the above preparation were hydrogenated in a Parr high pressure hydrogenation apparatus. 10 gms. Raney nickel catalyst were used and the hydrogenation carried out at 100°C. under 1700 lbs. pressure for 48 hours. The filtered aqueous solution was evaporated to a thick, sweet, colorless syrup which did not reduce Benedict's reagent and did not brown in hot 40% NaOH. Crystallization did not occur even after long standing at 0°C.

0.6 gms. of the hydrogenated material were mixed with 2 ml. of 50%

H_2SO_4 and 0.6 gms. benzaldehyde and set in the icebox. No crystalline benzal derivative was obtained.

This hydrogenated product had a methoxyl content of 4.6%. The unhydrogenated product had a methoxyl content of 1.3%.

0.1408 gms. of the hydrogenated material were acetylated with pyridine and acetic anhydride at 25°C. Acetic anhydride equivalent to 6.7 ml. of 0.5000 N. NaOH was taken up. 1 ml. of 0.5000 N. NaOH is equivalent to 0.5 millimols OH^-.

Reaction of Dihydroxy Maleic Acid with Formaldehyde

The formula of dihydroxy maleic acid is:

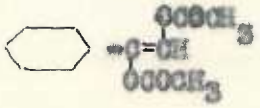
This compound was prepared by Fenton's method [15].

Solutions of dihydroxy maleic acid in excess 37% formaldehyde did not reduce 2,6 dichlorophenol indophenol.

Aqueous solutions of dihydroxy maleic acid evolved CO_2 even at low temperatures so that reaction with formaldehyde could not be established by this criterion.

The Action of Formaldehyde on Glycerophenylose Enediol Diacetate

Glycerophenylose enediol diacetate has the following structure:



This compound was prepared by the method of Reuben, Evans, and Meltzer [16].

100 gms. pure acetophenone were dissolved in 500 ml. dry ether and the solution cooled with ice. With vigorous stirring, 133.6 gms. bromine were added in the course of five minutes. After the color of bromine had disappeared the reaction mixture was poured into a large volume of mechanically stirred ice water. The ether solution was separated, washed with water, and dried over anhydrous Na_2SO_4, after which the solvent was removed in vacuo. The remaining syrup was crystallized by adding absolute ethanol. A yield of 100 gms. ω-bromo-acetophenone was obtained. This material possessed the melting point recorded in the literature, 50°C., and was strongly lachrymatory.

60 gms. ω-bromo-acetophenone and 50 gms. freshly-fused potassium acetate were dissolved in 450 ml. acetic anhydride in a three-necked liter flask, equipped with a mercury-sealed stirrer and a reflux condenser. After refluxing the mixture in an oil bath for three hours, it was allowed to stand for 48 hours at room temperature, then poured into mechanically stirred ice water. The brown oil that collected at the bottom of the flask was extracted with $CHCl_3$ and dried over anhydrous Na_2SO_4. When the $CHCl_3$ was removed in vacuo, a non-lachrymatory brown oil was left. Distillation of this oil, at 8 mm. Hg. yielded 10 gms. of a clear oil with a boiling point of 125°C. and a refractive index (25°C.) of 1.5260. This product reduced 2,6 dichlorophenol indophenol in the presence of pyridine and aqueous NaOH.

1.0 ml. of the pure glycerophenylose enediol diacetate obtained above was made up to 50.0 ml. with pure, dry pyridine.

1.0 ml. of the pyridine solution, when mixed with 2 ml. of 40% NaOH and 5 ml. pyridine, reduced 24.3 ml. of a saturated solution of 2,6 dichlorophenol indophenol.

1.0 ml. of the pyridine solution was mixed with 5 ml. of 37% formaldehyde and 5 ml. pyridine and allowed to stand for 30 minutes. Then 2 ml. of 40% NaOH were added. The mixture reduced 0.2 ml. of the same solution of indophenol.

1.0 ml. of the pyridine solution was mixed with 5 ml. of 37% formaldehyde, 5 ml. pyridine, and 2 ml. of 40% NaOH and allowed to stand 30 minutes. This mixture reduced 0.2 ml. of the same solution of indophenol.

The Effect of Formaldehyde on the Rotation of Glucose

1.000 gm. pure anhydrous d-glucose was dissolved in 37% formaldehyde, made up to a volume of 250 ml., and the optical rotation determined.

Day	Time	$[\alpha]_D^{23^\circ}$
1	10:30 AM	+87.0°
	11:00 AM	+76.0°
	4:30 PM	+82.0°
2	8:00 PM	+76.0°
3	4:30 PM	+81.2°
5	10:00 AM	+82.5°
9	10:00 AM	+83.7°
18	2:00 PM	+82.5°

The rotation of the same sample of glucose in water (same concentration) after mutarotation was $[\alpha]_D^{23^\circ}$ + 53.0°

The Effect of Formaldehyde on Enols

Samples of acetoacetic ester and acetyl acetone were titrated with dilute iodine solution and compared with the values obtained when these substances were mixed with formaldehyde. No difference could be noted, even when such mixtures were allowed to stand overnight.

Preparation of the Reaction Product of L-Ascorbic Acid and Formaldehyde

22.5 gms. ascorbic acid were dissolved in 50 ml. of 37% formaldehyde. The mixture was allowed to react in an atmosphere of nitrogen at 50°C. until reaction was complete (cessation of CO_2 formation). The mixture was diluted with water and repeatedly distilled under water pump vacuum at 30°C. until the distillate failed to form a precipitate with dimedon, at which point the reaction product was considered to be free of formaldehyde. The aqueous solution of the reaction product was evaporated to a thick syrup under water pump vacuum. Application of oil pump vacuum dried the syrup to a brittle, frothy mass which failed to crystallize even on long standing at 0°C. The color of the product varied from a light yellow to light brown. The complete operation described takes about one month. In all, sixteen preparations were made in this manner.

Attempts to remove excess formaldehyde by other means met with failure. The amount of excess formaldehyde was too great to remove conveniently with dimedon (dimethyl dihydro resorcinol) [17]. The dimedon appeared to react with the reaction product of formaldehyde and ascorbic acid to form a reddish brown syrup.

Addition of $NaHSO_3$ to the reaction mixture of formaldehyde and ascorbic acid was accompanied by decomposition and the liberation of much SO_2.

The removal of formaldehyde by H_2S, to form trithioformaldehyde, requires an acidity so high as to destroy the reaction product of ascorbic acid and formaldehyde.

A sample of the reaction mixture of ascorbic acid and excess formaldehyde was extracted in a liquid extractor with benzene, with chloroform,

and with ether. None of these solvents showed any appreciable formaldehyde content after several hours as judged by the dimedon test.

A sample of the reaction mixture of ascorbic acid and excess formaldehyde was evaporated to a heavy syrup in vacuo and dissolved in methyl cellosolve. Ether was added until the syrup separated. Several repetitions of this procedure did not free the syrup of formaldehyde.

In some cases it was found that removal of most of the water from the reaction mixture resulted in the precipitation of trioxymethylene. This could be removed by filtration. The filtrate from one such preparation was dried under water pump vacuum and then subjected to oil pump vacuum for several days. This method was unsatisfactory for the removal of formaldehyde.

Physical Properties of the Reaction Product of Ascorbic Acid and Formaldehyde

The data given below pertain only to the formaldehyde-free preparations made as described in the last section.

The reaction product is a yellow to yellow-brown syrup with a sweet taste and a characteristic pleasant odor. No sample has been crystallized. When very dry it is hard, brittle and quite hygroscopic.

The optical rotations of various preparations are given below. In each case the syrup was dried thoroughly in a vacuum desiccator. The concentration used was 4.0 gms. per 100 ml. No mutarotation was observed.

Preparation No.	$[\alpha]_D$	C.°
1	-16.6	27
3	-16.8	24
4	-15.0	23
5	-15.6	20
6	-15.0	20
7	-21.2	25
8	-17.1	20
9	-16.2	25

0.18 gms. of the reaction product of ascorbic acid and formaldehyde were dissolved in 10 ml. water. The pH, as measured with a glass electrode, was 3.6. One drop of 0.1 N. NaOH was added. The pH was found to be 5.2. Tap water was found to have a pH of 5.8.

Dry preparations of the reaction product are soluble in water, pyridine, methyl cellosolve, absolute methanol, and absolute ethanol. They are insoluble in ether, chloroform, dioxane, anisole, and benzene.

The refractive index of several preparations, as determined with an Abbé refractometer, varied from 1.5150 to 1.5202 at 25°C.

The molecular weight was determined by measuring the depression of the freezing point of water. 0.500 gms. glucose in 20.0 gms. water lowered the freezing point 0.28°C. The calculated molecular weight was 180. 0.500 gms. reaction product (dried in an Abderhalden drier at 30°C.) in 20.0 gms. water lowered the freezing point 0.85°C. The calculated molecular weight was 184. 0.7972 gms. reaction product in 25.0 gms. water lowered the freezing point 0.335°C. The calculated molecular weight was 187.

A glass molecular still was constructed in which the collecting surface was about 10 mm. above the surface of the substance to be distilled. Samples of the reaction product were dried in a vacuum desiccator, and then in an Abderhalden drier at 35°C. over P_2O_5. A sample was transferred to the molecular still which was then evacuated to a pressure of 0.0003 mm. Hg. No condensation took place up to a temperature of 75°C. at which point decomposition began.

Carbon and Hydrogen Determinations on the Reaction Product of Ascorbic Acid and Formaldehyde

The method of Ter Meulen was used.[19]

Sample 1 was dried in an Abderhalden drier at 70°C. over P_2O_5 for two hours.

Sample gms.	CO_2 gms.	H_2O gms.	Per cent C	Per cent H
0.0365	0.0532	0.0242	41.2	7.36
0.0351	0.0520	0.0229	40.4	7.25
0.0417	0.0617	0.0278	40.4	7.42
0.0417	0.0615	0.0276	40.1	7.36

Sample 2 was dried in an Abderhalden drier at 60°C. over P_2O_5 for 24 hours.

Sample gms.	CO_2 gms.	H_2O gms.	Per cent C	Per cent H
0.0400	0.0625	0.0243	42.6	6.75

Sample 3 was dried in an Abderhalden drier at 25°C. over P_2O_5 for 24 hours.

Sample gms.	CO_2 gms.	H_2O gms.	Per cent C	Per cent H
0.0460	0.0680	0.0265	41.2	6.50

Sample 4 was dried in an Abderhalden drier at 30°C. over P_2O_5 for four days.

Sample gms.	CO_2 gms.	H_2O gms.	Per cent C	Per cent H
0.0353	0.0543	0.0202	41.9	6.35

Pregl's method for the determination of carbon and hydrogen was also employed.

Sample 5 had been allowed to remain in an Abderhalden drier at 25°C. over P_2O_5 for several months.

Sample mgms.	CO_2 mgms.	H_2O mgms.	Per cent C	Per cent H
4.411	6.543	2.633	40.45	6.68
2.918	4.313	1.847	40.31	7.08

Qualitative Tests Applied to the Reaction Product of Ascorbic Acid and Formaldehyde

All experiments were performed on samples of syrup freed from formaldehyde in the manner previously described.

2,6 dichlorophenol indophenol was not reduced.

Barfoed's sugar reagent was reduced with difficulty.

The color was not returned to Schiff's reagent.

White and Green's aniline test yielded a yellow color, soluble in chloroform.

Seliwanoff's test for fructose yielded a claret color soluble in ethanol.

The Molisch test for carbohydrates was positive.

Development of blue or purple colors with $FeCl_3$ or sodium nitroprusside, a test for the enol group, was negative. In testing with $FeCl_3$ the sample was made just neutral to litmus with $NaHCO_3$ and a few drops of 1% $FeCl_3$ were added. The sodium nitroprusside test was performed in the same manner.

Biel's test for pentose was negative.

Kiliani's test for deoxy sugars was negative.

Rosenthal's test for methyl pentoses gave a positive cherry red color which was about one-third as strong as the control on xylose.

Oxidation with concentrated HNO_3 as in the mucic acid test yielded no insoluble acid or acid which forms an insoluble calcium or lead salt.

Aqueous solutions of the reaction product yielded no precipitate with 0.1 N. solutions of $BaCl_2$, $CdSO_4$, $CaCl_2$, $Pb(NO_3)_2$, or $Zn(NO_3)_2$.

Mieche [20] has published a method for the qualitative identification of sugars. It is based on the colors produced by various concentrations of phenol reagents. The following results were obtained with a 0.1 M. solution of the reaction product of ascorbic acid and formaldehyde.

Reagent	Color
Naphthol---------I	Brown
" ---------II	Yellow
" ---------III	Brown
Diphenylamine---I	Brown green
" ---II	Brown
" ---III	Green brown
Indole	Brown
Phloroglucinol	Brown

The above results are indicative of no known specific sugar.

When a 0.1 M. solution of the reaction product is underlaid with 0.3% β -naphthol in concentrated H_2SO_4, a green ring appears. This is a test for apiose, a branched chain sugar.

1 gm. of the reaction product was dissolved in 20 ml. HCl (sp. gr. 1.06), and distilled. A portion of the distillate was tested for furfural by adding aniline and acetic acid. No color was produced. Another portion of the distillate was tested for methyl furfural by adding a reagent consisting of 3 parts 95% ethanol and 1 part concentrated H_2SO_4. No color was produced.

Roe's method [21] was used to determine the action of HCl. The sample was treated with 6 N. HCl on the steam bath, cooled, and aniline acetate added. A brown color developed. Furfural produces a cherry red.

Potassium acetate forms insoluble complexes with certain sugars [22]. When concentrated alcoholic solutions of potassium acetate and the reaction

product were mixed, no precipitate formed.

Connor's test [23] for active hydrogen groups was negative.

Methylene ethers of sugars have been shown to split out formaldehyde on treatment with NH_4OH [24]. About 0.1 gm. of the reaction product was treated with concentrated NH_4OH for several hours. The solution was acidified and dimedon was added. No precipitate was observed.

A compound such as hydroxymethylene benzoin [25] splits off formaldehyde when treated with alcoholic NaOH. About 0.1 gm. of the reaction product of ascorbic acid and formaldehyde was heated for three minutes with alcoholic NaOH. This solution did not return the color to Schiff's reagent.

In the test for formaldehyde with phloroglucinol in 1 : 1 HCl, the reaction product of ascorbic acid and formaldehyde decomposed to a tar and did not form a true precipitate.

The reaction product of ascorbic acid and formaldehyde was oxidized slowly by selenium dioxide as evidenced by the formation of elementary selenium.

0.2 gms. of the reaction product of ascorbic acid and formaldehyde were dissolved in 50 ml. of 0.1 N. H_2SO_4. The mixture was steam distilled. To the distillate was added a reagent ($NaAc-HgCl_2$) for formic acid. No precipitate was formed.

0.2 gms. of the reaction product of ascorbic acid and formaldehyde were dissolved in 35 ml. of 10% H_2O_2 and heated at 100°C. for three hours. When the $NaAc-HgCl_2$ reagent for formic acid was added, a precipitate formed.

Kumler [26] has shown that a positive iodine atom may be introduced

into certain compounds such as acetoacetic acid. When the reaction product of ascorbic acid and formaldehyde was treated with iodine and H_2O_2 by Kumler's method, no iodine was taken up.

The reaction product of ascorbic acid and formaldehyde formed no crystalline derivative with phenylhydrazine, 2,4 dinitro phenylhydrazine, p-bromo phenylhydrazine, p-nitro phenylhydrazine, methyl phenylhydrazine, hydroxylamine, or dimedon.

Link's method [27] for preparing benzimidazole derivatives from aldoses was applied to the reaction product of ascorbic acid and formaldehyde. 10 gms. of the syrup in methanol were oxidized with hypoiodite. No potassium salt precipitated, but 2.6 gms. of a barium salt were obtained. When this was condensed with o-phenylene diamine, the mixture became quite dark and could not be decolorized with charcoal. On adding NH_4OH a brownish gelatinous precipitate formed. An attempt to purify this by forming the copper salt resulted in the formation of a few milligrams of a white substance which was found to be inorganic in nature.

The Reducing Power of the Reaction Product of Ascorbic Acid and Formaldehyde

The reducing power was determined by the Shaffer-Hartman method [28], using reagent number 50. A solution of the reaction product of ascorbic acid and formaldehyde was made which contained 0.4 mg. per ml. 5 ml. of this solution reduced copper equivalent to 7.05 ml. of 0.005 N. $Na_2S_2O_3$. This is equivalent to 0.79 mg. glucose (7.05 x 0.113). 0.79 ÷ 2 = 0.395. Therefore, the reducing power of the reaction product of ascorbic acid and formaldehyde toward this reagent is 39.5% of that of glucose.

Hypoiodite Oxidation of the Reaction Product of Ascorbic Acid and Formaldehyde

The method used was that of Kline and Acree [29].

A sample of the sugar is titrated with 0.1 N. NaOH or HCl until it is neutral to phenolphthalein. 5 ml. of 0.1 N. iodine is added from a burette, then 7.5 ml. of 0.1 N. NaOH is added, drop by drop, from a burette. This process is repeated until 22 ml. of the iodine and 35 ml. of the NaOH have been added. Two minutes are allowed for completion of oxidation. The solution is acidified with 0.1 N. HCl to liberate iodine from any iodate present and the liberated iodine titrated with 0.1 N. $Na_2S_2O_3$. The excess acid is titrated with 0.1 N. NaOH.

In the following experiments dry, formaldehyde-free samples of the reaction product of ascorbic acid and formaldehyde were used.

Sample gms.	0.1000 N. I_2 ml.	0.1000 N. NaOH ml.
0.5649	15.02	20.02
0.7040	16.82	23.10
0.9925	20.02	25.06

The formula for calculating percentage of aldose in the sample is

$$\frac{\text{wt. of millimol}}{\text{wt. of sample}} \times \frac{\text{ml. reagent used}}{\text{ml. reagent required per millimol}}$$

One millimol of a hexose requires 20 ml. of 0.1 N. iodine and 30 ml. of 0.1 N. NaOH. Thus, using the data in the table above:

Sample gms.	Per cent aldose	
	I_2 consumption	NaOH consumption
0.5649	24.0	22.2
0.7040	21.6	19.7
0.9925	18.2	15.6

Determination of Active Hydrogen in the Reaction Product of Ascorbic Acid and Formaldehyde

Zerewitinoff's method [30] was used. A Grignard reagent was prepared

from 2 gms. magnesium ribbon, 7 gms. methyl iodide, and 20 ml. iso-amyl ether. This was allowed to react in a Zerewitinoff apparatus and the methane formed was measured in a Schiff nitrometer. A blank was run on the reagent.

0.1059 gms. of the reaction product of ascorbic acid and formaldehyde were dissolved in 5 ml. pure, dry pyridine and allowed to react with 5 ml. of the Grignard reagent. 56.7 ml. (corr.) methane were formed.

13.2 ml. of hydrogen correspond to the presence of one active hydrogen in 0.1059 gms. of compound: (0.1059 ÷ 180) x 22,400 = 13.2. The active hydrogen atoms per mol of compound were accordingly found to be 56.7 ÷ 13.2 = 4.3.

In two more experiments the results were as follows:

Sample gms.	Methane ml.	Methane per H ml.	Active H per mol
0.0985	52.6	12.3	4.2
0.0997	48.0	11.2	4.3

Acetylation of the Reaction Product of Ascorbic Acid and Formaldehyde

The method used was that of Peterson and West [31].

0.1951 gms. of the reaction product of ascorbic acid and formaldehyde were weighed into a 16 x 150 mm. test tube. 5.00 ml. of an acetylating mixture (one part acetic anhydride and two parts pure, dry pyridine) were added. The mixture was stoppered and shaken until the syrup was dissolved, then put in the refrigerator for 24 hours. At the end of this time the sample was poured into 200 ml. ice water and titrated with 0.500 N. NaOH. The acetic anhydride taken up was equivalent to 7.5 ml. of this NaOH. 1 ml. of 0.5 N. NaOH = 0.5 mM. OH^- and 7.5 ml. of 0.5 N. NaOH = 3.75 mM. OH^-. The sample taken was roughly one millimol.

Benzoylation of the Reaction Product of Ascorbic Acid and Formaldehyde

5 gms. of the reaction product of ascorbic acid and formaldehyde were dissolved in 40 gms. pure, dry pyridine and cooled in an ice bath. 20 gms. benzoyl chloride were added slowly with good stirring. The mixture was allowed to stand in the ice box over night. The crystalline precipitate of pyridine hydrochloride was filtered off and the remaining liquid was diluted with $CHCl_3$. The solution was washed with dilute H_2SO_4 to remove pyridine, then with dilute $NaHCO_3$ to remove benzoic acid, and finally with water. The washed $CHCl_3$ solution was dried over anhydrous Na_2SO_4, decolorized with charcoal, and evaporated in a vacuum. The syrup was taken up in ether. This solution was allowed to evaporate slowly. No crystallization occurred even on long standing in the refrigerator. The syrup had an aromatic smell characteristic of benzoylated compounds and when dry was quite brittle.

Methoxyl Determinations on the Reaction Product of Ascorbic Acid and Formaldehyde

The procedure used was that of Zeisel as modified by Elek [3]. The apparatus of Elek was used.

The sample was weighed into a small glass cup on a Kuhlmann microbalance. The cup was then dropped into the reaction vessel and the sample dissolved in a few drops of acetic anhydride. The washing chamber was filled with 0.5 ml. of 5% $Na_2S_2O_5$ and 0.5 ml. of 5% $CdSO_4$. The absorption tube was charged with eight drops of bromine in 10 ml. of 10% potassium acetate in glacial acetic acid.

A platinum bead and 2 ml. pure hydriodic acid (specific gravity 1.7)

were added to the reaction vessel, which was then connected to the absorption tube. Pure CO_2 from a Kipp generator was run through the apparatus at a rate such that only one bubble of gas was in the absorption vessel at any instant. After 15 minutes, the contents of the reaction vessel were heated to boiling with a microburner and kept at a slow boil for two hours. At the end of this time the contents of the absorption tube were washed into a 125 ml. Erlenmeyer flask containing 10 ml. of 15% aqueous potassium acetate. Formic acid was added to destroy excess bromine, complete removal being taken as the point at which methyl red was no longer decolorized. 2 ml. of 10% KI and 5 ml. of N. H_2SO_4 were added and the iodine liberated was titrated with N/30 $Na_2S_2O_3$.

Sample mg.	N/30 $Na_2S_2O_3$ ml.
Blank	0.08
5.357	0.10
7.582	0.12

These results indicate the absence of methoxyl groups in the material.

Periodic Acid Oxidation of the Reaction Product of Ascorbic Acid and Formaldehyde

The method used was essentially that of Voris, Ellis, and Maynard [33].

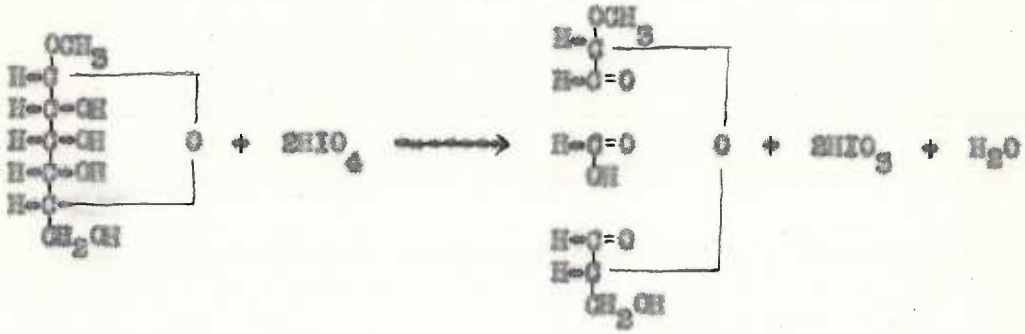
The sample to be oxidized was dissolved in an aqueous solution of periodic acid and 20-30 minutes allowed for complete reaction. The reaction mixture was neutralized, after the addition of three drops of 15% $MgSO_4$, by adding dilute NaOH dropwise until a faint cloudiness appeared. 0.1 N. H_2SO_4 was then added until the cloudiness just disappeared. To the reaction mixture was added 10 ml. of a phosphate buffer containing 12 gms.

$Na_2HPO_4 \cdot 12H_2O$ and 4.2 ml. of 10 N. H_2SO_4 per 100 ml. solution. 2 ml. of 10% KI were added and liberated iodine was titrated with 0.1 N. $Na_2S_2O_3$. It has been found by Rappaport et al. [34] that iodine liberated from periodate by KI can be titrated, in the presence of iodate, by $Na_2S_2O_3$ if the pH is held between 4.4 and 7 by a buffer. In this oxidation periodate is reduced to iodate.

Periodic acid has been shown by Malaprade [35] to oxidize adjacent hydroxyl groups only. Terminal hydroxyl groups are oxidized to formaldehyde, while those in between two other hydroxyl groups are oxidized to formic acid.

For example:

Oxidation with periodic acid has been used extensively in structural studies. Another example of the action of periodic acid is given below.



Thus, the use of periodic acid makes it possible to determine the number of contiguous hydroxyl groups and ring position, as well as the position of other substituents.

The amount of periodate unused is determined by reaction with KI.

$$HIO_4 + 2KI + H_2O \longrightarrow HIO_3 + I_2 + 2KOH$$

The iodine formed is then titrated with $Na_2S_2O_3$.

$$2Na_2S_2O_3 + I_2 \longrightarrow Na_2S_4O_6 + 2\,NaI$$

Thus: $HIO_4 \equiv 2Na_2S_2O_3$.

0.18 gms. dry, formaldehyde-free reaction product of ascorbic acid and formaldehyde were weighed into a small beaker and 20.00 ml. of approximately 0.2 M. HIO_4 were added. When titrated, the excess HIO_4 used 19.20 ml. of 0.1000 N. $Na_2S_2O_3$.

20.00 ml. HIO_4 were equivalent to 80.40 ml. of 0.1000 N. $Na_2S_2O_3$. Thus, 80.40-19.20 = 61.20 ml. of $Na_2S_2O_3$ is equal to the amount of HIO_4 that reacted with the sample. 61.20 ÷ 80.40 = 0.76. Since 20.00 ml. of approximately 0.2 M. HIO_4 are equivalent to 4 millimols of the acid, and 76% of this was used up, then one millimol of the syrup reacted with 3 millimols of HIO_4.

0.0804 gms. of the dry, formaldehyde-free reaction product of ascorbic acid and formaldehyde were weighed into a small beaker. 7 ml. of approximately 0.2 M. HIO_4 were added. In about 30 minutes concentrated $Ba(NO_3)_2$ was added, drop by drop, until no more BaI_2 precipitated. The mixture was filtered and to the filtrate was added 1 ml. of 10% KI and 1 ml. of N. H_2SO_4. The iodine liberated was titrated with 0.1 N. $Na_2S_2O_3$. To this solution were added 30 ml. saturated dimedon solution. The mixture was heated to 100°C for two hours and allowed to stand overnight. The precipitate was collected on a Gooch crucible, dried, and weighed. The weight of the precipitate was 0.1127 gms. The melting point of this crystalline precipitate was 136°C. The melting point of the dimedon derivative of formaldehyde is recorded in the literature [17] as 139°C. The weight of formaldehyde represented by the precipitate was 0.1127 x 0.1027 = 0.0116 gms. 0.44 millimols of syrup (0.0804 ÷ 0.18) yielded

0.39 millimols of formaldehyde (0.0116 ÷ 0.03).

0.1791 gms. of dry, formaldehyde-free reaction product of ascorbic acid and formaldehyde were dissolved in 20.00 ml. of approximately 0.2 M. HIO_4. After allowing one hour for reaction, the mixture was neutralized to the methyl red endpoint [36] with 0.1 N. NaOH. 61.20 ml. of 0.1047 N. NaOH were required. 20.00 ml. of the approximately 0.2 M. HIO_4 required 40.80 ml. of 0.1047 N. NaOH for titration. Thus during the reaction, acid was formed equivalent to 61.20 - 40.80 = 20.40 ml. of 0.1047 N. NaOH. The oxidation of one millimol of the syrup produced 2 millimols of acid, supposedly formic.

0.1795 gms. of dry, formaldehyde-free reaction product of ascorbic acid and formaldehyde were weighed into a beaker and 20.00 ml. of approximately 0.2 M. HIO_4 were added. After letting the mixture react for two hours, most of the iodate and periodate ions were removed by precipitation as strontium salts by adding a hot concentrated solution of $Sr(OH)_2$, drop by drop. The mixture was filtered, 1 ml. of 10% KI and 1 ml. of N. H_2SO_4 added, and the iodine liberated was titrated with 0.1 N. $Na_2S_2O_3$. This mixture was steam distilled. The distillate was collected until a 50 ml. aliquot contained so little acid that it could not be determined by titrating with 0.1 N. NaOH. About a liter of distillate was collected. The acidity of the distillate was equivalent to 25.75 ml. of 0.1047 N. NaOH. The mixture was then made acid to congo red with acetic acid and 50 ml. of formic acid reagent (containing 50 gms. $HgCl_2$ and 27.5 gms. sodium acetate per liter) were added. The whole was heated on a steam bath for five hours and allowed to stand overnight. The precipitated HgCl was collected on a Gooch crucible, dried, and weighed. The precipitate

weighed 0.8111 gms. This precipitate represents 0.0793 gms. formic acid (0.8111 x 0.0977) or 1.7 millimols (0.0793 ÷ 0.046) produced from one millimol of syrup. This result indicates the presence of at least four adjacent hydroxyl groups.

Oxidation of the Reaction Product of Ascorbic Acid and Formaldehyde by Sodium Periodate

0.027 gms. of dry, formaldehyde-free reaction product of ascorbic acid and formaldehyde were allowed to react for an hour with 20.00 ml. of 0.0303 M. sodium periodate. The excess periodate, determined as described previously for periodic acid, was equivalent to 0.80 ml. of 0.1000 N. $Na_2S_2O_3$. The sodium periodate used was equivalent to 3.10 ml. of 0.1000 N. $Na_2S_2O_3$. Thus, (3.10 - 0.80) ÷ 3.10 = 0.74, or, 74% of the periodate was used. The sample of syrup used contained 0.027 ÷ 0.18 = 0.15 millimols. The periodate that reacted with the syrup represented 0.46 millimols. One millimol of syrup reacted with three millimols of periodate, which checks with the oxidation by periodic acid.

Lead Tetraacetate Oxidation of the Reaction Product of Ascorbic Acid and Formaldehyde

The lead tetraacetate used was prepared by the method of Cooper and Deasy [37].

The reaction is carried out in a three-necked flask fitted with a thermometer, stirrer, and gas inlet tube. A mixture of 300 ml. glacial acetic acid and 150 ml. acetic anhydride is heated to 65°C. and a slow stream of dry chlorine led in with stirring. About 120 gms. red lead are introduced in five equal portions, each addition being delayed until the

color of the previous portion has faded. The temperature during the reaction is held at 65°-80°C. When reaction is complete, the hot mixture is filtered through a heated funnel. Lead tetraacetate crystallizes from the filtrate upon cooling. This product, about 90% pure, was used without further purification since the contaminants (lead diacetate, lead chloride, acetic acid) do not interfere with its use as an oxidizing agent.

It has been found by Criegee [38] that lead tetraacetate in a non-aqueous medium oxidizes polyhydric alcohols as does periodic acid. For example:

The amount of lead tetraacetate unused is determined by reaction with KI.

The iodine formed is then titrated with $Na_2S_2O_3$.

Thus: $PbAc_4 \equiv 2Na_2S_2O_3$

Oxidation with lead tetraacetate was carried out as described by Criegee [38].

0.0639 gms. dry, formaldehyde-free reaction product of ascorbic acid and formaldehyde were weighed into a small flask and 20.00 ml. of 0.0935 M. lead tetraacetate in glacial acetic acid added. About ten minutes were allowed for reaction. 30 ml. of a buffer iodide solution, containing 20 gms. KI and 300 gms. anhydrous sodium acetate per liter of solution, were added. The iodine liberated was equivalent to 20.72

ml. of 0.1000 N. $Na_2S_2O_3$. 20.00 ml. of 0.0935 M. lead tetraacetate is equivalent to 37.40 ml. of 0.1000 N. $Na_2S_2O_3$. Then, (37.40 - 20.72) ÷ 37.40 = 0.446 of the original amount of lead tetraacetate. This is equal to 0.00187 ÷ 0.446 = 0.834 millimols. The sample of syrup used contained 0.0639 x 0.18 = 0.355 millimols. Thus, one millimol of syrup reacted with 2.35 millimols of lead tetraacetate.

0.2983 gms. dry, formaldehyde-free reaction product of ascorbic acid and formaldehyde were dissolved in 25 ml. water. A saturated solution of lead tetraacetate in glacial acetic acid was added slowly with stirring until the appearance of a brown precipitate, indicating complete reaction. Enough normal H_2SO_4 was added to precipitate the lead in solution, the mixture was filtered, and the filtrate diluted to 250 ml. with water. A 50 ml. aliquot was mixed with 20 ml. saturated dimedon solution, heated on the steam bath for two hours, and allowed to stand overnight. The precipitate was collected on a weighed Gooch crucible, dried and weighed. The precipitate weighed 0.0315 gms. and had a melting point of 185°C. If the whole filtrate had been used, 0.263 gms. precipitate would have been obtained. This amount of precipitate represents 0.027 gms. formaldehyde (0.263 x 0.1027). Thus, 1.65 millimols syrup (0.2983 ÷ 0.18) yielded 0.9 millimols formaldehyde (0.027 ÷ 0.03).

The Reaction of Iodic Acid with the Reaction Product of Ascorbic Acid and Formaldehyde

It was found that the reaction product rapidly liberated iodine from iodic acid at room temperature. The iodine liberated could be determined only if an excess of iodic acid were avoided.

In the following experiment the solution of the reaction product of ascorbic acid and formaldehyde was mixed with the iodic acid solution, CCl_4 was added and the mixture was shaken vigorously. The CCl_4 layer was separated, washed with a little water, and the iodine titrated with $Na_2S_2O_3$.

Reaction product ml. (0.2072 gms. in 10.0 ml.)	0.1000 N. HIO_3 ml.	0.0100 N. $Na_2S_2O_3$ ml.
1.00	2.0	1.00
0.50	2.0	0.60
1.00	1.0	1.07
0.50	1.0	0.65
1.00	2.0	1.40
+ 2 ml. of 2.5 N. H_2SO_4		

Thus, as a maximum, 0.115 millimole of syrup liberated 0.014 millimole of iodine from iodic acid.

Permanganate Oxidation of the Reaction Product of Ascorbic Acid and Formaldehyde

5.6 gms. dry, formaldehyde-free reaction product of ascorbic acid and formaldehyde were dissolved in 60 ml. water, 30 ml. of 5 N. H_2SO_4 added, and the mixture titrated with normal $KMnO_4$. After about 200 ml. $KMnO_4$ had been added, an aliquot of 5 ml. was taken to which were added 10 ml. of a saturated dimedon solution. In a short time a precipitate appeared in the dimedon mixture. This precipitate was filtered off and dried. The melting point was 186°C. The mixed melting point with a sample of the dimedon derivative of formaldehyde was 186°C. The melting point of the dimedon derivative of formaldehyde is 189°C. The original reaction mixture was further titrated with $KMnO_4$ which now was taken up very slowly. In all, 513 ml. of normal $KMnO_4$ were reduced before the end-

point was reached. Only at the beginning of the experiment did a sample of the reaction mixture yield a precipitate with dimedon. The reaction mixture was concentrated to about 200 ml. in a vacuum at 50°C. The distillate was tested for formic acid by adding $HgCl_2$ and sodium acetate. No precipitate formed.

Paget and Berger [39] have devised a test for ascorbic acid based on the fact that it yields oxalic acid when oxidized with $KMnO_4$.

To 1 ml. of 0.1% ascorbic acid were added a little dilute acetic acid and 0.5 ml. normal $KMnO_4$. Dilute H_2O_2 was added to destroy the excess $KMnO_4$. 0.5 ml. concentrated HCl and a little zinc were added and the mixture was put on the steam bath for three minutes. When cool, five drops 1% phenylhydrazine hydrochloride were added, the mixture heated to boiling and cooled rapidly. An equal volume of concentrated HCl and five drops 8% potassium ferrocyanide were added. A cherry red color, the test for oxalic acid, was produced.

When treated as above, a purified sample of the reaction product of ascorbic acid and formaldehyde gave the same result except that the color was a little lighter than in the case of ascorbic acid.

Glycogenic Power of the Reaction Product of Ascorbic Acid and Formaldehyde

The method used for determining liver glycogen was essentially that devised by Somogyi [40].

Two groups of four white rats each were fasted 24 hours before the experiment. At the beginning of the experiment each of the four test animals was given 2 ml. of a 25% solution of the reaction product of ascorbic acid and formaldehyde by stomach tube. Three hours later this was repeated, so that each test animal received a total of 1 gm. of

the reaction product of ascorbic acid and formaldehyde. Three hours after the second feeding, both the test and control animals were decapitated with a large shears, the liver was immediately removed, quickly ground in a small chopper and added to a prepared tube, the whole process being completed in at least 60 seconds. The prepared tube referred to was a shallow-pointed-tip centrifuge tube of 50-ml. capacity to which had been added 2 ml. of 30% KOH and then weighed to the nearest milligram. The liver was introduced into these tubes in such a way that no particles adhered to the sides of the tube. The tubes containing the liver samples, about 1 gm., were reweighed. If more than 1 gm. of liver had been taken, additional 30% KOH was added to maintain the ratio of 1 gm. liver to 2 ml. of 30% KOH. The tubes were heated about 30 minutes on a steam bath. When cool, a volume of ethanol equal to 1.2 times the volume of KOH was added to precipitate the glycogen. The tubes again were heated to boiling, cooled, and centrifuged. The supernatant liquid was decanted. The tubes were drained for a few minutes and then wiped dry with filter paper. 15 ml. of 0.6 N. HCl were added to the tubes which then were heated for two hours on the steam bath. When cool, the solution was neutralized to a phenol red endpoint with 0.5 N. NaOH and then diluted to 50 ml. The glucose in these samples was determined by the Shaffer-Hartman method [28] using reagent number 50.

Group I - Control Animals

Rat No.	Liver taken gms.	Glycogen mg.	Gms. glycogen per 100 gms. liver
1	2.575	3.70	1.144
2	1.786	2.28	0.130
4	2.782	0.55	0.236
26	2.791	2.62	0.094 (ave. 0.151)

Group II - Test Animals

Rat No.	Liver taken gms.	Glycogen mgms.	Gms. Glycogen per 100 gms. liver
18	1.691	4.60	0.260
10	1.057	7.75	0.734
9	2.373	9.76	0.412
35	1.427	2.26	0.158 (ave. 0.391)

It should be noted that the test animals had diarrhea during the experiment.

Methylation of the Reaction Product of Ascorbic Acid and Formaldehyde

The first method used was that of Emil Fischer for methyleting free sugar groups.

26.4 gms. dry, formaldehyde-free reaction product of ascorbic acid and formaldehyde were dissolved in 20 ml. absolute methanol. To this was added a solution of dry HCl in absolute methanol. Absolute methanol was added until the final volume was 375 ml. and the concentration of HCl was 0.33%. The mixture was refluxed for two hours. The HCl was removed by treating the solution with Ag_2CO_3 and the methanol was removed at the water pump at 25°C. About 16 gms. of a dark brown syrup were obtained. This material reduced Benedict's reagent in the cold. The methoxyl content, determined in the manner described previously, was 10.0%. The refractive index was 1.5170 at 25°C.

A sample was oxidized with HIO_4 as described previously. 0.1900 gms. of the methylated product (roughly 1 millimol) were mixed with 20.00 ml. of approximately 0.2 N. HIO_4. When titrated the excess HIO_4 used 46.60 ml. of 0.1000 N. $Na_2S_2O_3$. 20.00 ml. HIO_4 were equivalent to 80.40 ml. of 0.1000 N. $Na_2S_2O_3$. Thus, 80.40 - 46.60 = 33.80 ml. of $Na_2S_2O_3$ are

equal to the amount of HIO_4 that reacted with the sample. 33.60 ÷ 80.40 = 0.417. Since 20.00 ml. of 0.2 M. HIO_4 represents 4 millimols, and 41.7% of this was used, then roughly 1 millimol of the methylated product reacted with 1.6 millimols of HIO_4.

0.027 gms. of the methylated product were allowed to react for an hour with 80.00 ml. of 0.0308 M. (0.616 millimols) sodium periodate. The excess periodate, determined as described previously, was equivalent to 1.90 ml. of 0.1000 N. $Na_2S_2O_3$. The sodium periodate used was equivalent to 3.10 ml. of 0.1000 N. $Na_2S_2O_3$. Thus, (3.10 - 1.90) ÷ 3.10 = 0.387 or, 38.7% of the periodate reacted. The sample of syrup used represented approximately 0.029 ÷ 0.196 = 0.15 millimols. 0.24 millimols of periodate reacted with the 0.15 millimols of syrup.

0.19 gms. of the methylated product were oxidized with HIO_4 and the solution was tested for formaldehyde in the manner previously described. The formation of a precipitate (with dimedon) of the proper melting point showed the presence of formaldehyde.

0.1974 gms. of the methylated product were oxidized with 20 ml. of approximately 0.2 M. HIO_4 and the formic acid formed was determined as described previously. The total acid produced was equivalent to 28.75 ml. of 0.1047 N. NaOH. The HgCl precipitate weighed 0.8215 gms. which represented 0.0803 gms. formic acid (1.7 millimols) formed from about 1 millimol of syrup.

8.4 gms. of the methylated product were dissolved in 50 ml. water, 10 gms. Raney catalyst added, and the mixture introduced into a Parr high pressure hydrogenation apparatus. Reaction was carried out at 100°C. for

48 hours under 1450 lbs. hydrogen pressure. The mixture was filtered and the water removed at 50°C. at the water pump. The methoxyl content of a sample dried in an Abderhalden drier at 30°C. was 6.4%.

1 gm. of the methylated product was dissolved in 30 ml. acetone containing 6 gms. anhydrous $ZnCl_2$. The mixture was shaken for 24 hours at 25°C., then poured into a solution of 7 gms. anhydrous K_2CO_3 in 7 ml. water overlaid with 30 ml. ether. The whole was shaken for half an hour and then filtered. The solvents were evaporated slowly at the water pump. No crystals appeared. A rather labile syrup was finally obtained.

Methylation of the above methylated product was continued by the method of West and Holden [41]. A large distilling flask is fitted with a sealed stirrer and an extra neck through which passes a dropping funnel. The delivery tube of the flask is fitted with a condenser attached to a suction flask. The reaction flask is immersed in a water bath. The sample, about 0.14 mol of a hexose, for example, is dissolved in 15 ml. water. This is introduced into the flask. The stirrer is started and the water bath heated to 55°C. A mixture of 90 ml. methyl sulfate and 125 ml. CCl_4 is run into the flask, 400 ml. of 40% NaOH is added at the rate of one drop per two seconds for five minutes, then one drop per second for five minutes, then three drops per second until the distillation of CCl_4 ceases. The remainder of the alkali is added rapidly and the temperature of the water bath is raised to 70°-75°C. and maintained there during the rest of the reaction. 160 ml. methyl sulfate now are added at 3-5 drops per second. After all the methyl sulfate has been added the water bath is heated to boiling for 30 minutes. The contents of the flask are cooled, water is added to dissolve separated Na_2SO_4.

and the mixture is extracted four times with 150 ml. portions of $CHCl_3$. The $CHCl_3$ extract is dried with anhydrous Na_2SO_4 and the $CHCl_3$ removed at the water pump.

6.2 gms. of the methylated product were further methylated by the method described above. About 1 gm. of a rather fluid brown syrup was obtained. The methoxyl content, determined as described previously, was 45.8%.

This material was treated with ether. It was found that most of the material was soluble. Further methylation was accomplished by the method of Freudenberg and Nixon [42]. To the ether solution was added 1 gm. of sodium ribbon. The mixture was shaken vigorously for four hours and then allowed to stand at 25°C. for 24 hours. The solution was now decanted into 10 ml. methyl iodide in a distilling flask. The solvents were removed at the water pump at 25°C. until a paste remained. 25 ml. methyl iodide were added, the mixture was shaken for four hours and allowed to stand overnight. 30 ml. absolute ether were added. When the NaI had crystallized out, the solution was filtered and the ether was removed at the water pump at 40°C. About 0.6 gms. of a light yellow liquid were obtained. The methoxyl content was 46.5%. This material did not reduce Benedict's reagent.

In another experiment, Purdie's reagents were used for methylation. 1 gm. of the product obtained by methylation with methanolic HCl was dissolved in the least possible quantity of pure dry dioxane (dried and distilled over sodium). 25 gms. methyl iodide and 2 gms. Ag_2O were added and the mixture was refluxed for eight hours. The mixture was filtered

and concentrated to a syrup at the water pump. This material was not soluble in ether so the process was repeated. A portion of this material, soluble in ether, was further methylated by the method of Freudenberg and Nixon described above. The product had a methoxyl content of 29% and did not reduce Benedict's reagent. The yield was 0.5 gms.

Preparation of the Acetone Derivative of the Reaction Product of Ascorbic Acid and Formaldehyde

The method of Fischer and Beer [43] was used in preparing the acetone derivative. 80 gms. freshly-fused $ZnCl_2$ are dissolved in 300 ml. pure dry acetone. The solution is decanted from any insoluble material and to this solution are added about 10 gms. of the sugar. The mixture is shaken until the sugar is in solution and allowed to stand for 12 hours. The mixture is poured into a solution of 70 gms. K_2CO_3 in 70 ml. of water overlaid with 300 ml. ether. This mixture is shaken vigorously for one-half hour. The solution is decanted from the $ZnCO_3$ residue which is washed twice with 100 ml. 1:1 acetone and ether. The washings are added to the main solution and the whole evaporated at the water pump at 40°C.

10 gms. dry, formaldehyde-free reaction product of ascorbic acid and formaldehyde were treated as above. Evaporation at the water pump was carried out very slowly and stopped as soon as crystals began to appear. The solution was allowed to stand for 24 hours, the crystals were filtered off, and the filtrate concentrated again, repeating the process until no more crystals formed. If the crystals were not separated in this way it was found that they redissolved when the solvents were gone and the heavy syrup formed would not crystallise. Further,

acetone, ether, or petroleum ether could not be used to dissolve the mother liquor away from the crystals. Best results were obtained by draining the crystals on porous tile and recrystallizing them from hot water. The yield of recrystallized material was about 0.2 gms. The crystals were slightly yellowish white needles melting at 235°C. with decomposition. The compound was slightly soluble in water, acetone, petroleum ether, dioxane, methanol, chloroform and insoluble in ether. Benedict's reagent was readily reduced.

About 50 mgms. crystals were dissolved in 5 ml. of H_2SO_4 and heated on the steam bath for 15 minutes. The solution was neutralized with $Ba(OH)_2$, filtered, and evaporated at the water pump at 25°C. A small amount of a clear, glassy material was obtained which could not be crystallized and which reduced Benedict's reagent.

50 mgms. crystals were mixed with 0.2 gms. phenylhydrazine hydrochloride and 0.3 gms. anhydrous sodium acetate in 2 ml. water and the whole was heated on the steam bath for an hour. On cooling and standing overnight no crystals were formed.

20 mgms. crystals were dissolved in 5 ml. of a saturated solution of dimedon slightly acidified with acetic acid. The solution was heated on the steam bath for an hour and allowed to stand overnight. No crystals were observed.

20 mgms. of the crystals were dissolved in 5 ml. of a solution of phloroglucinol in 1 : 1 HCl (test for formaldehyde). No precipitate was formed.

Acetone Determinations on the Acetone Derivative

The micro method of Bell and Harrison [44] was employed in determining the acetone content.

The sample, containing about 1 mgm. acetone, is dissolved in 5 ml. of N. H_2SO_4 and introduced into a special steam distillation apparatus. The receiver is charged with 25 ml. of 0.01 N. iodine and 5 ml. of N. NaOH. The mixture in the reaction vessel is steam distilled for a half hour. The alkaline iodine solution is then acidified with 7 ml. of N. H_2SO_4 and the excess iodine determined by titration with 0.01 N. $Na_2S_2O_3$.

The following determinations were made on a sample of the recrystallized acetone derivative.

Sample mg.	0.0100 N. I_2 ml.	0.0100 N. $Na_2S_2O_3$ ml.	Per cent acetone
2.515	25.00	17.22	31.0
2.542	25.00	17.20	30.9

Acetone determinations on the syrupy material left after crystallization were:

2.060	25.00	18.45	33.1

Determination of the Molecular Weight of the Acetone Derivative

Rast's camphor method [45] was used.

Into a capillary tube 50 mm. long and 2 mm. in diameter are weighed about a mgm. of the sample and about 20 mgms. camphor. The tube is sealed, fastened to a thermometer, and immersed in a phosphoric acid bath. The mixture in the tube is melted and shaken to obtain homogeneity, cooled and heated again very slowly. The point at which the last particle melts is considered the melting point. The molal freezing point depression of the

camphor used must be determined. This was done by making several determinations with pure diacetone mannitol. The molal freezing point depression was found to be 40.0°C., the value most often cited in the literature.

Molecular weight determinations made on a sample of the recrystallized acetone derivative were:

Sample mgms.	Camphor mgms.	ΔC°	Molecular weight
0.842	9.134	16.3	228
0.997	19.539	9.9	230
1.163	23.042	8.7	231

Carbon and Hydrogen Determinations on the Acetone Derivative

Carbon and hydrogen values were obtained by the Pregl micro method.

A recrystallized sample of the acetone derivative was used and the following results were obtained:

Sample mgms.	H_2O mgms.	CO_2 mgms.	Per cent H	Per cent C
3.784	2.293	7.775	6.72	55.9
4.682	2.933	9.590	6.71	55.8
4.965	2.966	10.137	6.63	55.6

Calculation of the empirical formula:

H 6.7 ÷ 1 = 6.7
C 55.8 ÷ 12 = 4.6
O 37.4 ÷ 16 = 2.3

The empirical formula is then C_2H_3O. $C_{10}H_{15}O_5$ would have a molecular weight of 225.

Optical Rotation of the Acetone Derivative

A 2 dm. micro polariscope tube with a capacity of about 0.2 ml.

was used. Determinations were made on a concentrated water solution and on a concentrated dioxane solution. No rotation could be observed in either case.

Oxidation of the Acetone Derivative with Periodic Acid

30.6 mgms. of the recrystallized acetone derivative were allowed to react with 10.00 ml. of 0.1951 N. HIO_4. The excess HIO_4 was equivalent to 29.30 ml. of 0.1011 N. $Na_2S_2O_3$. The total amount of HIO_4 used was equivalent to 38.60 ml. of 0.1011 N. $Na_2S_2O_3$. Thus, (38.60 - 29.30) ÷ 38.60 = 0.24 or 24% of the HIO_4 was reduced. This is equivalent to 0.47 millimols. The sample of acetone derivative represented 0.13 millimols. Thus, one millimol of the acetone derivative reacted with 3.6 millimols of HIO_4.

Preparation of the Semicarbazone of the Reaction Product of Ascorbic Acid and Formaldehyde

5 gms. semicarbazide hydrochloride and 7 gms. sodium acetate were dissolved in 25 ml. water. To this solution were added 5 gms. of the reaction product of ascorbic acid and formaldehyde. The mixture was warmed to insure complete solution, filtered, and allowed to stand overnight. The resultant crystalline precipitate was filtered off. The mother liquor was concentrated to about half the volume by allowing it to stand on top of an oven for a few hours, then set aside to crystallize. The total yield of crude material was 0.5 gms. When recrystallized from water the derivative was obtained as small colorless plates melting at 242°C. The compound reduced hot Benedict's reagent and liberated iodine from iodic acid.

About 50 mgms. of the crystals were hydrolyzed by heating with 1 ml. concentrated HCl at 100°C. for five minutes. The hydrolysate reduced hot

Benedict's reagent. No precipitate was formed when dimedon was added, indicating the absence of formaldehyde.

Determination of Nitrogen in the Semicarbazone

Nitrogen was determined by the method of Ter Meulen [18].

In this method the sample is heated in the presence of hydrogen and a catalyst. The nitrogen present is converted to ammonia which is titrated with standard acid.

A 30-50 mgm. sample of the nitrogenous substance is mixed thoroughly with finely powdered activated nickel. A boat, containing the mixture, is introduced into a combustion tube which contains a packing, about 25 cm. long, of activated nickel and asbestos. The portion of the tube containing the catalyst is heated to 350°C. during the determination. Pure dry hydrogen is passed through the apparatus and the exit gases are allowed to escape through a U-shaped vessel containing about 15 ml. water and 2 drops of Tashiro's indicator. During the determination the ammonia formed is continuously titrated with standard acid. Thus the progress of the combustion can be followed easily. The sample is heated with a microburner at such a rate that the whole analysis is accomplished in about 1.5 hours.

Recrystallized samples of the semicarbazone yielded the following:

Sample mgms.	N/14 H_2SO_4 ml.	Per cent N
40.4	8.27	20.5
34.1	7.00	20.5
28.7	5.85	20.4

Determination of Carbon and Hydrogen in the Semicarbazone

The Pregl micromethod was used.

Sample mgms.	H_2O mgms.	CO_2 mgms.	Per cent H	Per cent C
4.400	2.450	5.395	6.19	33.4
3.824	2.159	4.655	6.25	33.2
3.735	2.075	4.579	6.17	33.4

Calculation of the empirical formula follows:

C	33.4	÷ 12	= 2.8
H	6.2	÷ 1	= 6.2
O	39.9	÷ 16	= 2.5
N	20.5	÷ 12	= 1.46

These data indicate the formula $C_2H_4O_{1.8}N$. The molecular formula $C_6H_{12}O_5N_3$ would have a molecular weight of 207.

Determination of the Molecular Weight of the Semicarbazone

The molecular weight was determined by measuring the elevation of the boiling point of acetic acid. Other methods could not be used because the material was insoluble in camphor, cold water, hot dioxane, and pinene dibromide. The apparatus used was similar to that of Beckmann except that the liquid was internally heated with a coil of platinum wire (electrically heated) and the lower part of the apparatus was fitted into a vacuum bottle.

0.2250 gms. of the recrystallized semicarbazone were dissolved in 20.8 gms. pure acetic acid. The first and second observations of the elevation of the boiling point were 0.155°C. and 0.165°C. respectively, corresponding to molecular weights of 214 and 201. The solution darkened appreciably during the procedure.

Hydrogenation of the Reaction Product of Ascorbic Acid and Formaldehyde

All hydrogenations were carried out in a Parr high pressure hydrogenation apparatus and Raney nickel was used as catalyst.

It was found that hydrogenation at 2000 lbs. hydrogen pressure and

150° C. for 24 hours was the most satisfactory procedure in that complete reduction was obtained with a minimum of by-products. At temperatures lower than 150° C. the reaction proceeded slowly. At temperatures appreciably above 150° C. an oily substance was formed which had a mint-like odor. If the reaction mixture was left in contact with the nickel catalyst for more than about 24 hours the hydrogenated product seemed to retain small amounts of catalyst in a colloidal form, coloring the product green. This colloidal nickel could not be removed with decolorizing charcoal or by heating. Only colorless preparations were used for analysis.

In order to determine the quantity of hydrogen necessary for complete reduction, 36.2 gms. of dry formaldehyde-free reaction product of ascorbic acid and formaldehyde were dissolved in water and 10 gms. Raney catalyst added. The total volume was 105 ml. Hydrogenation was carried out at 150° C. for 24 hours. During this time the pressure dropped from 2090 lbs. to 1890 lbs., a difference of 200 lbs. Since the material no longer reduced Benedict's reagent it was assumed that reduction was complete. The pressure drop per mol of hydrogen for this apparatus and a volume of 105 ml. is 945 lbs. Since 0.21 mol of material were used, the theoretical drop would be 0.21 x 945 = 198 lbs. if each mol of the material reduced took up one mol of hydrogen.

In preparing the hydrogenated product it was found that it was unnecessary to use a purified sample of the reaction product of ascorbic acid and formaldehyde. Mixtures of ascorbic acid and formaldehyde, when hydrogenated under the above conditions, yielded a product indistinguishable from that prepared from the purified material. The identical prop-

erties of the hydrogenated products obtained by both methods of preparation were shown as follows:

Experiment A - 22.5 gms. ascorbic acid were dissolved in 50 ml. of 37% formaldehyde. The mixture was allowed to stand for an hour and then hydrogenated. The product was methylated with methyl sulfate as described previously. and then with sodium and methyl iodide as described previously. The final methylated product was purified by vacuum distillation.

Experiment B - 22.5 gms. ascorbic acid were dissolved in 50 ml. of 37% formaldehyde and allowed to react for five days in nitrogen at 50° C. The excess formaldehyde was removed by vacuum distillation as previously described. The reaction product was then hydrogenated and the hydrogenated material methylated as in Experiment A.

	Experiment A gms.	Experiment B gms.
Ascorbic acid used	22.5	22.5
Yield on hydrogenation	17.8	17.4
Yield on methylation with methyl sulfate	8.8	8.9
Yield on methylation with sodium and methyl iodide	4.4	4.4
Properties of the purified methylated product:		
Boiling point range at 0.025 mm. Hg.	40-55° C.	40-55° C.
Refractive index at 20° C.	1.4383	1.4370
Methoxyl content	55%	55%
Optical rotation	0	0

Physical Properties of the Hydrogenated Reaction Product of Ascorbic Acid and Formaldehyde

This material was a colorless syrup which could not be crystallized. It was soluble in water, pyridine, absolute methanol, and absolute ethanol; insoluble in ether, chloroform, and benzene.

A 10% solution had a pH of 5.5.

The refractive index of various samples was between 1.4965 and 1.5000 at 25°C.

The optical rotation of an aqueous solution containing 1.000 gms. substance per 100 ml. was $[\alpha]_D^{23^\circ}$ + 5.2°.

Fractionation of about 5 gms. of syrup was accomplished by vacuum distillation in the molecular still described previously. In one experiment distillation was carried out at a pressure of 0.003 mm. Hg. Fraction 1 was cut from 20° - 80° C. Fraction 2 was cut from 80° - 110° C. Above 110° C. the compound began to turn yellow and distillation was discontinued.

	Refractive index	Gms.
Fraction 1	1.4730	0.3
Fraction 2	1.4920	0.5
Residue	1.4920	4.0

Molecular Weight of the Hydrogenated Reaction Product of Ascorbic Acid and Formaldehyde

The molecular weight was determined by measuring the depression of the freezing point of water. 0.5297 gms. glucose in 20 gms. water lowered the freezing point 0.275° C. The calculated molecular weight was 179. 0.5520 gms. of the hydrogenated reaction product (dried in an Abderhalden drier at 20° C.) in 20 gms. water lowered the freezing point 0.275° C. The

calculated molecular weight was 150.

Carbon and Hydrogen Determinations on the Hydrogenated Reaction Product of Ascorbic Acid and Formaldehyde

The method of Ter Meulen [18] was used. Samples were prepared by drying in an Abderhalden drier under oil pump vacuum at 100°C. for 24 hours.

Sample gms.	H_2O gms.	CO_2 gms.	Per cent H	Per cent C
0.0351	0.0229	0.0520	7.35	40.4
0.0417	0.0279	0.0617	7.42	40.4
0.0417	0.0276	0.0613	7.35	40.1

Calculation of the empirical formula:

H 7.35 ÷ 1 = 7.35
C 40.4 ÷ 12 = 3.36
O 52.25 ÷ 16 = 3.26

Although a simple whole number ratio is not obtained, the results indicate the empirical formula CH_2O.

Qualitative Tests Applied to the Hydrogenated Reaction Product of Ascorbic Acid and Formaldehyde

Benedict's reagent was not reduced.

Treatment with hot 40% NaOH did not cause browning or charring.

Oxidation with 50% HNO_3 as in the mucic acid test did not result in the formation of an insoluble derivative.

When a solution of the hydrogenated product was poured carefully on to a 0.05% solution of β-naphthol in concentrated H_2SO_4 a green ring was formed. This test is given by apiose [46], a branched chain hydroxymethyl aldopentose.

5 gms. of the hydrogenated product were dissolved in 15 ml. of 50% H_2SO_4 and 5 gms. pure benzaldehyde added. The mixture was allowed to stand in the ice box for several days. A syrup was obtained which failed to crystallize.

Methoxyl Determinations on the Hydrogenated Reaction Product of Ascorbic Acid and Formaldehyde

Methoxyl values were determined by the modified Zeisel procedure described previously.

The samples were prepared for analysis by drying in an Abderhalden drier at 25°C. for several weeks.

Preparation	Sample mgms.	N/30 $Na_2S_2O_3$ ml.	Per cent methoxyl
1	5.151	3.80	12.7
2	4.033	2.80	11.8
3	5.777	2.64	12.1

The following results were obtained on the fractions from the vacuum distillation previously described.

Fraction	Sample mgms.	N/30 $Na_2S_2O_3$ ml.	Per cent methoxyl
1	4.151	4.60	18.6
	3.909	4.25	18.7
2	5.256	4.05	15.3
residue	4.831	2.55	9.1

Acetylation of the Hydrogenated Reaction Product of Ascorbic Acid and Formaldehyde

In the first series of experiments acetylation was carried out by the method of Peterson and West previously described.

The samples were prepared by drying in a vacuum desiccator for

several days.

Sample A was prepared by hydrogenating the purified reaction product of ascorbic acid and formaldehyde. Sample B was prepared by hydrogenating a mixture of ascorbic acid and formaldehyde.

Experiment 1 - reaction for six days at 5°C.

Sample	Sample gms.	0.5000 N. NaOH ml.	mM. OH^-
A	0.2049	9.1	4.55
B	0.2120	10.5	5.25

Experiment 2 - reaction for six days at 25°C.

Sample	Sample gms.	0.5000 N. NaOH ml.	mM. OH^-
A	0.2156	9.7	4.85
B	0.2071	10.2	5.10

In a second series of experiments acetylation was carried out according to the method of Hafner, Swinney, and West [47].

Experiment 3 - reaction for 1.25 hours at 100°C.

Sample	Sample gms.	0.5000 N. NaOH ml.	mM. OH^-
A	0.1915	9.33	4.67
B	0.1925	9.48	4.74

During these experiments no insoluble derivative was noted when the acetylating mixture was poured into water.

Determination of Hydroxyl Groups in the Hydrogenated Reaction Product of Ascorbic Acid and Formaldehyde

A modified Karl Fischer reagent specific for water, has been prepared by Smith, Bryant, and Mitchell [48]. One-liter quantities of the reagent are prepared by dissolving 84.7 gms. U.S.P. iodine in a mixture

of 269 ml. pure dry pyridine and 667 ml. pure dry methanol. This solution is cooled with ice and 64 gms. liquid SO_2 added. The reagent was stored in and dispensed from a reservoir burette.

The reagent is standardized as follows: A 10 ml. sample of pure dry methanol is titrated with the reagent to a permanent brown endpoint. Then 18 ml. water are weighed into a liter volumetric flask, diluted to about a liter with the same supply of methanol titrated, and adjusted to volume in a thermostat at 25.0°C. 10 ml. samples of this standard solution are then titrated. The titration of the standard solution minus 99% of the blank titration on the methanol is the amount of Karl Fischer reagent equal to the weight of water taken.

10.00 ml. of the methanol used had a titration value of 1.60 ml. Karl Fischer reagent.

10.00 ml. of the standard solution, containing 0.1799 gms. water had a titration value of 59.75 ml. 59.75 - 1.57 = 58.18 ml. Karl Fischer reagent which is equivalent to 0.1799 gms. water. Then 323 ml. Karl Fischer reagent is equivalent to 1.000 gms. water.

Using this reagent Bryant, Mitchell, and Smith [49] worked out a method for determining alcoholic hydroxyl groups. 5 to 10 ml. of sample are weighed into a 100 ml. glass stoppered volumetric flask about one-third filled with pure dry dioxane. The mixture is made up to volume with more dioxane, shaken until homogeneous and adjusted to volume after standing at least 30 minutes in a thermostat at 25.0°C. 5 ml. are transferred to a 250 ml. glass-stoppered flask and 20 ml. catalyst solution added. The catalyst solution is made by dissolving 100 gms. boron trifluoride gas and 2 ml. water in sufficient glacial acetic acid to make one liter of

solution. The flask containing the sample and a control containing 20 ml. catalyst and 5 ml. dioxane are tightly stoppered and placed in a water bath at 67.0°C. for two hours. At the end of this time the flasks are removed and allowed to cool. 5 ml. pure pyridine are added to each and the mixtures titrated with Karl Fischer reagent.

5.985 gms. absolute ethanol were mixed with dioxane as above and a 5.00 ml. sample treated as indicated. The 5.00 ml. sample had a titration of 67.3 ml. Karl Fischer reagent. The blank titration was 40.6 ml. 67.3 - 40.6 = 26.7 ml. The strength of the Karl Fischer reagent at this point had fallen slightly so that 338 ml. was equivalent to 1.000 gms. water. 26.7 ÷ 338 = 0.0790 gms. water. 0.392 x weight of alcohol equals the weight of water produced by esterification. The 5.00 ml. sample taken contained 0.1992 gms. alcohol. 0.1992 x 0.392 = 0.0785 gms. water produced theoretically.

0.7156 gms. of the hydrogenated reaction product of ascorbic acid and formaldehyde (dried thoroughly in an Abderhalden drier) were dissolved in 100 ml. glacial acetic acid and adjusted as indicated above. This material was not soluble in dioxane. 5.00 ml. of this solution, when treated as above, had a titration value of 45.1 ml. The control had a titration value of 39.0 ml. 45.1 - 39.0 = 6.1 ml. 366 ml. Karl Fischer reagent was equivalent to 1.000 gms. water. 6.1 ÷ 366 = 0.0167 gms. water. Assuming five hydroxyl groups per molecule, one mol of the hydrogenated product would yield 90 gms. water. The 5.00 ml. acetic acid solution contained 0.0358 gms. hydrogenated product which would yield 0.5 x 0.0358 or 0.0179 gms. water.

Periodic Acid Oxidation of the Hydrogenated Reaction Product of Ascorbic Acid and Formaldehyde

These experiments were carried out by the methods described previously.

0.182 gms. dry hydrogenated reaction product of ascorbic acid and formaldehyde were allowed to react for a few minutes with 20.00 ml. of approximately 0.2 M. HIO_4. When titrated, the excess HIO_4 used 16.40 ml. of 0.1000 N. $Na_2S_2O_3$. 20.00 ml. of the HIO_4 were equivalent to 80.40 ml. of 0.1000 N. $Na_2S_2O_3$. Thus 80.40 - 16.40 = 64.00 ml. $Na_2S_2O_3$ are equal to the amount of HIO_4 that reacted with the sample. The fraction of HIO_4 reacting was 64.00 ÷ 80.40 = 0.79. Since 20.00 ml. of the HIO_4 represents 4 millimols, and 79% of this was used, then one millimol of the sample reacted with 3.2 millimols of HIO_4.

0.1691 gms. dry hydrogenated product were oxidized with 20 ml. of approximately 0.2 M. HIO_4 and the formaldehyde formed was determined by the dimedon procedure described previously. 0.2361 gms. of formaldehyde dimedon compound were obtained, corresponding to 0.0242 gms. formaldehyde. 0.0242 ÷ 0.03 = 0.81 millimols of formaldehyde were obtained from 0.94 millimols (0.1691 ÷ 0.18) of hydrogenated product.

When the hydrogenated product was oxidized with HIO_4 the odor of acetaldehyde was quite noticeable. An analysis for this compound was made by the method of Nicolet and Shinn [50].

By the use of this method acetaldehyde can be determined in the presence of formaldehyde. The reaction is carried out in an aeration apparatus consisting of three 2.5 x 20 cm. test tubes connected with

glass tubing. The first tube serves as a reaction vessel, the second and third tubes serve as absorption vessels. The acetaldehyde formed in the first tube is carried into the second and third tubes by a stream of CO_2. In the first tube are placed the sample, 0.2 gms. alanine, 5 ml. of N. $NaHCO_3$, and 10 ml. of 0.1 N. sodium arsenite. In the second tube are placed 5.00 ml. of 2% sodium metabisulfite diluted to 25 ml. with water. In the third tube are placed 3.00 ml. of 2% sodium metabisulfite diluted to 25 ml. with water. The apparatus is connected, 1-2 ml. of 0.5 M. HIO_4 are added to the first tube from a dropping funnel containing CO_2 under pressure and CO_2 is passed through the apparatus at a rate of one liter per minute for an hour. The contents of tubes 2 and 3 are pooled and the excess bisulfite is destroyed by adding 0.1 N. iodine until a blue endpoint is obtained with starch. The blue color is destroyed by adding saturated $NaHCO_3$ solution and the bound bisulfite is determined by titrating with 0.02 N. iodine. 1 ml. of 0.02 N. iodine is equivalent to 1.64 mgms. methyl pentose.

18.0 mgms. of the hydrogenated reaction product were treated as described and yielded acetaldehyde equivalent to 3.30 ml. of 0.0202 N. iodine. 3.30 x 1.64 = 5.4 mgms. methyl pentose. 30.3 mgms. of the hydrogenated reaction product yielded acetaldehyde equivalent to 5.60 ml. of 0.0202 N. iodine. 5.60 x 1.64 = 9.18 mgms. methyl pentose.

0.1867 gms. the dry hydrogenated reaction product were allowed to react with 20.00 ml. of approximately 0.2 M. HIO_4 for one hour. The mixture was neutralized to the methyl red endpoint with 0.1047 N. NaOH. 58.68 ml. were required. 20.00 ml. of the HIO_4 required 40.80 ml. of

0.1047 N. NaOH. Thus, during the reaction acid was formed equivalent to 56.68 - 40.80 = 15.88 ml. of 0.1047 N. NaOH. Therefore, one millimol of the hydrogenated product produced about one and a half millimols of acid.

0.1804 gms. the dry hydrogenated reaction product were allowed to react with 20 ml. of approximately 0.2 M. HIO_4 and the formic acid produced was determined as described previously. 24.25 ml. of 0.1047 N. NaOH were required to neutralize the distillate. The HgCl precipitate weighed 0.5431 gms., corresponding to 0.0531 gms. or 1.15 millimols formic acid.

Sodium Periodate Oxidation of the Hydrogenated Reaction Product of Ascorbic Acid and Formaldehyde

0.027 gms. dry hydrogenated product were allowed to react for one hour with 20.00 ml. of 0.0303 M. sodium periodate. The excess periodate was equivalent to 1.40 ml. of 0.1000 N. $Na_2S_2O_3$. The sodium periodate used was equivalent to 3.10 ml. of 0.1000 N. $Na_2S_2O_3$. Thus (3.10 - 1.40) ÷ 3.10 = 0.55 or, 55% of the periodate was used. The sample represented 0.15 millimols (0.027 ÷ 0.18). The periodate that reacted represented 0.33 millimols.

Lead Tetraacetate Oxidation of the Hydrogenated Reaction Product of Ascorbic Acid and Formaldehyde

0.1265 gms. the dry hydrogenated product were allowed to react with 40.00 ml. of 0.0330 M. lead tetraacetate in glacial acetic acid. The excess lead tetraacetate liberated iodine equivalent to 29.20 ml. of 0.1000 N. $Na_2S_2O_3$. The lead tetraacetate added was equivalent to

71.20 ml. of 0.1000 N. $Na_2S_2O_3$. Thus 71.20 - 29.20 = 42.00 ml. of 0.1000 N. $Na_2S_2O_3$ is equivalent to the tetraacetate reduced. The fraction used was 42.00 ÷ 71.20 = 0.59. 0.59 x 0.00366 = 2.7 millimols. The sample of hydrogenated product represented 0.7 millimols.

The Acetone Derivative of the Hydrogenated Reaction Product of Ascorbic Acid and Formaldehyde

10 gms. the dry hydrogenated product were allowed to react with acetone in the presence of anhydrous $ZnCl_2$ as described previously. A light brown liquid, soluble in petroleum ether, was obtained and distilled at a pressure of 0.1 mm. Hg. Most of the material came over at about 59°C.

Acetone determinations yielded the following results.

Sample mgms.	0.1000 N. $Na_2S_2O_3$ ml.	Acetone mgms.	Per cent acetone
1.796	6.55	0.682	38.0
2.310	6.27	0.862	37.4

Glycogenic Power of the Hydrogenated Reaction Product of Ascorbic Acid and Formaldehyde

The details of the procedure and the dosages have been given previously. Since this experiment was done at the same time, the same control group was used.

Group III - Test Animals

Rat No.	Liver gms.	Glycogen mgms.	Gms. Glycogen per 100 gms. liver
27	1.815	8.50	0.460
37	2.010	14.90	0.740
25	2.318	4.75	0.205
12	2.031	7.05	0.347 ave. 0.438

Methylation of the Hydrogenated Reaction Product of Ascorbic Acid and Formaldehyde

16.0 gms. dry hydrogenated product were methylated with methyl sulfate in the presence of NaOH by the method of West and Holden [41]. 7 gms. of a light yellow non-reducing syrup were obtained. The methoxyl content of the product was 46.33%

This methylated product was further methylated with methyl iodide and Ag_2O under reflux for 8 hours. A syrup was obtained having a methoxyl content of 50.66%.

In another experiment, 28.4 gms. dry hydrogenated product were methylated twice with methyl sulfate and NaOH. 20 gms. of a non-reducing syrup, having a methoxyl content of 62.9%, were obtained. A sample of this material was distilled at a pressure of 0.8 mm. Hg. Fraction 1 consisted of material that distilled up to 112°C. Fraction 2 was constant boiling at 112°C. Fraction 3 was material that distilled between 113° and 118°C. All fractions were water - white liquids.

Fraction	Sample mgms.	N/30 $Na_2S_2O_3$ ml.	Per cent methoxyl	$n^{25°}$
1	1.468	5.45	64.12	1.4442
2	1.498	5.75	66.16	1.4459
3	1.783	6.87	66.40	1.4460

About 1 mg. of the above methylated product (not distilled) was refluxed with 10 ml. of 7% HCl for an hour. The product was extracted with CCl_4 and the extract was dried with anhydrous Na_2SO_4. The CCl_4 was removed at the water pump and the product dried in an Abderhalden drier. The methoxyl content was 60.3%. The material did not reduce Benedict's reagent.

Montgomery, Hann, and Hudson [81] have made sugar acetals by the

following method. The hemi-acetal of the sugar is first prepared. From this the chloro derivative is made by treatment with anhydrous $AlCl_3$. This product is treated with methyl alcohol and Ag_2O to obtain the acetal.

1 gm. of the methylated product (not distilled) was dissolved in dry alcohol-free CCl_4. The mixture was cooled to 5°C. and 1 gm. anhydrous $AlCl_3$ added. After 30 minutes ice water was added and the CCl_4 layer separated. This solution was dried with anhydrous Na_2SO_4 and the solvent removed at the water pump. The syrup obtained was dissolved in pure dry methanol and Ag_2O added. After shaking for five minutes, the mixture was filtered, the solvent removed at the water pump, and the syrup obtained was dried in an Abderhalden drier. The methoxyl content was 63.4% as compared with 62.9% methoxyl in the untreated syrup.

0.24 gms. of the methylated product (not distilled) were allowed to react with 10 ml. of approximately 0.2 N. HIO_4. The excess HIO_4 was equivalent to 36 ml. of 0.1000 N. $Na_2S_2O_3$. 10 ml. of the HIO_4 used was equivalent to 40.20 ml. of 0.1000 N. $Na_2S_2O_3$. This demonstrated the absence of adjacent free hydroxyl groups.

54 gms. dry hydrogenated reaction product of ascorbic acid and formaldehyde were methylated twice with methyl sulfate and NaOH. 24.7 gms. of a methylated product were obtained. A sample of this material was distilled at a pressure of 0.5 mm. Hg.

Methoxyl determinations were run on the fractions as shown below.

Fraction	Boiling range C.°	Sample mgms.	N/30 $Na_2S_2O_3$ ml.	Per cent methoxyl
1	75-90	1.991	5.41	56.9
2	90	1.660	5.90	61.5
3	90-120	1.678	5.90	60.6

This material was pooled, methylated again with methyl sulfate and

NaOH, and further with methyl iodide and Ag_2O. When this product was distilled the following results were obtained.

Fraction	Boiling range C°	mm. Hg.	$n^{25°}$	$[\alpha]_D^{25°}$	Per cent methoxyl	Yield ml.
1	65-70	0.038	1.4400	-	63.39	1-2
2	75	0.038	1.4420	-	62.24	1-2
3	75-83	0.038	1.4430	-		1-2
4	83-84	0.038	1.4433	-	62.00	1-2
5	80-80	0.027	1.4443	-		1-2
6	80	0.027	1.4452	-	59.80	1-2
7	80-95	0.027	1.4472	-		5
8	95-97	0.027	1.4489	-		1-2

Fraction 7 was remethylated with methyl iodide and sodium. When this preparation was distilled at a pressure of 0.07 mm. Hg. the boiling range was 80°-90° C. The refractive index at 25° C. was 1.4440 and the methoxyl content was 66.90%.

All of the above fractions (1 to 8) were pooled and remethylated with methyl iodide and sodium. The product was distilled at a pressure of 0.1 mm. Hg. Two arbitrary fractions were taken. Fraction 1 had the boiling range 50°-77° C. Fraction 2 had the boiling range 77°-82° C., most of it coming over at 80°-82° C. About 8 ml. of each fraction were obtained.

Fraction	$n^{20°}$	Per cent methoxyl
1	1.4392	66.8
2	1.4448	66.8

Molecular Weight of the Methylated Hydrogenated Reaction Product of Ascorbic Acid and Formaldehyde

The molecular weight was determined by measuring the freezing point depression of benzene. 0.6991 gms. methylated hydrogenated material ($n^{25°}$ = 1.4440 and 66.8% methoxyl) were dissolved in 17.56 gms. dry benzene.

The freezing point depression was 0.810°C. The calculated molecular weight was 245.

Carbon and Hydrogen Determination on the Methylated Hydrogenated Reaction Product of Ascorbic Acid and Formaldehyde

Carbon and hydrogen were determined by the Pregl micromethod.

Determination of carbon and hydrogen on fraction 1 ($n^{25°}$ = 1.4392 and 66.8% methoxyl).

Sample mgms.	H_2O mgms.	CO_2 mgms.	Per cent H	Per cent C
4.178	3.802	8.126	9.24	53.0
4.057	3.371	7.875	9.20	52.9

Determination on fraction 2 ($n^{25°}$ = 1.4448 and 66.8% methoxyl).

Sample mgms.	H_2O mgms.	CO_2 mgms.	Per cent H	Per cent C
3.595	3.090	7.061	9.80	53.6
3.900	3.319	7.678	9.47	53.6

Calculation of the empirical formula is as follows:

H	9.5	÷ 1	=	9.5
C	53.5	÷ 12	=	4.46
O	37.0	÷ 16	=	2.3

This indicates the approximate empirical formula of C_2H_4O. The molecular weight of the substance (245) suggests a possible molecular formula of $C_{12}H_{24}O_6$.

CONCLUSIONS

The Reaction between Ascorbic Acid and Formaldehyde

Reaction between ascorbic acid and formaldehyde has been proven by showing: (1) a change in optical rotation of mixtures of ascorbic acid and formaldehyde, (2) the evolution of carbon dioxide, from mixtures of ascorbic acid and formaldehyde, in amounts proportional to the amount of ascorbic acid present, (3) an effect of formaldehyde on the pK value of ascorbic acid, (4) the disappearance of the indophenol and iodine reducing power of ascorbic acid upon treatment with formaldehyde, (5) that ascorbic acid can not be recovered from mixtures of ascorbic acid and formaldehyde which have had an opportunity to react in an inert atmosphere.

The position of reaction in the ascorbic acid molecule is the enediol group. This is indicated by the facts that: (1) formaldehyde reacts with all enediol compounds tested when judged by the above criteria, (2) the indophenol reducing power of ascorbic acid and related compounds, which is blocked by formaldehyde, resides in the enediol group.

That the reaction of ascorbic acid and formaldehyde is not simple, but complex, can be concluded from two facts. First, formaldehyde blocks the indophenol reducing power of ascorbic acid rapidly at room temperature but carbon dioxide is not evolved from the mixture until much later. Second, the change in optical rotation does not follow a simple curve. Initially there is a sharp rise followed by a slow drop.

From these facts the reaction between ascorbic acid and formaldehyde may be assumed to take place in two steps. First, formaldehyde reacts with the enediol group of ascorbic acid, forming a labile intermediate product. Second, this intermediate product breaks down into carbon dioxide and a

final reaction product.

The Nature of the Reaction Product of Ascorbic Acid and Formaldehyde

The reaction product is a mixture as indicated by the facts that: (1) at least two derivatives have been obtained which do not arise from the same parent compound, (2) the product does not show a whole number ratio of carbon, hydrogen, and oxygen, (3) the failure of the product to crystallize points to a mixture, (4) the hydrogenated reaction product may be fractionated by vacuum distillation.

The reaction product is sugar-like in that: (1) it gives the Molisch test for carbohydrates, (2) it is derived from ascorbic acid which in turn is a sugar derivative, (3) it is a polyhydroxy reducing substance, (4) it tastes sweet.

Qualitative tests permit the following conclusions. The reaction product is not one of the well-defined hexoses or pentoses. This is shown by the fact that it does not yield furfural or methyl furfural when boiled with 12% HCl nor does it give any of the fully characteristic color reactions of such sugars. Further, all specific tests for the common aldoses and ketoses are negative. The extreme lability of the reaction product in both acid and basic solutions makes these tests of little value. Only two tests are definitely positive, that for a methyl pentose and that for apiose (a branched chain sugar).

Although the reaction product readily reduces alkaline copper reagents no free aldehyde group is present since it does not return the color to Schiff's reagent.

At least one of the components of the reaction product possesses a

carbonyl group since it is possible to obtain a semicarbazone derivative. This component may be the aldose fraction indicated by hypoiodite oxidation.

Tests with ferric chloride and sodium nitroprusside indicate the absence of an enol structure.

No free carboxyl group is present since the reaction product is essentially neutral in reaction.

No evidence has been obtained to show that formaldehyde adds to ascorbic acid in a well-known manner. Compounds containing the methylene ether group yield formaldehyde upon hydrolysis with ammonium hydroxide. The formaldehyde can be detected by Schiff's reagent. Such compounds also give a test for formaldehyde when treated with an acid phloroglucinol reagent. Hydroxymethylene compounds such as hydroxymethylene benzoin split off formaldehyde when treated with sodium hydroxide. The formaldehyde can be detected by Schiff's reagent. When the above tests were applied to the reaction product of formaldehyde and ascorbic acid no formaldehyde could be detected. Further, the number of hydroxyl groups present indicates that formaldehyde does not add in a manner that would produce a hydroxyl group.

An unsaturated linkage is not present because neither iodine nor bromine was reduced. A conjugated system is ruled out since the product added only one molecule of hydrogen.

At least one component seems to have a ring structure since hydrogenation produced a methoxyl group.

Acetylation of the reaction product of ascorbic acid and formalde-

hyde indicated four hydroxyl groups. Methylation also indicated four hydroxyl groups. A methylated product containing 46.5% methoxyl was obtained. The methoxyl value for four methoxyls is 52.6%. From the amounts of formaldehyde and formic acid produced by periodic acid or lead tetraacetate oxidation it may be concluded that one hydroxyl group is primary, two are secondary, and that all four hydroxyls are adjacent. One hydroxyl group is extraordinary in that it is very easily methylated at room temperature.

One component of the reaction product of ascorbic acid and formaldehyde possesses a very labile grouping which reduces iodic acid. Since the semicarbazide derivative is the only derivative which has this property it must be derived from the iodic acid reducing component. The presence of this component somewhat vitiates the quantitative aspects of the periodic acid oxidation.

That a methyl group is present in some component is suggested by the fact that positive results were obtained with Rosenthal's test for methyl pentoses and that the hydrogenated reaction product yielded acetaldehyde when oxidized with periodic acid.

The average molecular weight of the components in the reaction product is 184-187.

Carbon-hydrogen analyses indicate a mixture of compounds, the major portion of which has the formula $C_6H_{12}O_6$.

	Per cent C	Per cent H	Molecular weight
$C_6H_{12}O_6$	40.0	6.67	180
Results obtained	40.1-42.6	6.35-7.35	184-187

Nature of the Hydrogenated Reaction Product of Ascorbic Acid and Formaldehyde

Hydrogenation of the reaction product of ascorbic acid and formaldehyde resulted in the addition of one molecule of hydrogen. This hydrogenated product contained about 12% methoxyl. When distilled in a vacuum it yielded a fraction containing 18.6% methoxyl. The theoretical value for one methoxyl is 16%.

Acetylation indicated the presence of five hydroxyl groups. Methylation resulted in products with a maximum methoxyl content of 56.8%. The theoretical value for five methoxyls is 62.0%.

When the hydrogenated product was oxidized with periodic acid, formaldehyde, acetaldehyde, and formic acid were produced. The amount of formaldehyde obtained indicates one primary hydroxyl group per molecule. Acetaldehyde production indicates that about 30% of the material contains a methyl group. The formic acid obtained indicates an average of one and a half secondary hydroxyl groups per molecule.

The fact that no crystalline benzal derivative was obtained speaks against the presence of a simple sugar alcohol since nearly all these compounds form such derivatives.

Since the hydrogenated product gives a positive test for apiose, which is a hydroxymethyl branched chain carbohydrate, this type of structure may be present.

The average molecular weight of the components in the hydrogenated product was 180.

Carbon-hydrogen analyses indicate a mixture with the following formulas as limits.

	Per cent C	Per cent H	Molecular weight
$C_6H_{14}O_6$	39.6	7.68	182
$C_6H_{12}O_5$	43.8	7.32	164
Results obtained	40.4	7.35	180

Nature of the Acetone Derivative of the Reaction Product of Ascorbic Acid and Formaldehyde

This derivative has an acetone content of 31.0%. The theoretical value for one acetone group is 25.7%. It is felt that during the acetone determination some reactive group breaks to yield a substance which behaves like acetone toward the alkaline iodine reagent employed. This substance is not formaldehyde since acid hydrolysis has been shown to produce none.

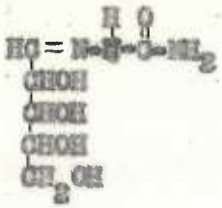
Periodic acid oxidation indicates the presence of at least three adjacent hydroxyl groups.

Molecular weight determinations and carbon-hydrogen analyses check well and indicate a compound with the formula $C_{10}H_{18}O_5$. The parent compound would have the formula $C_7H_{11}O_5$, and a molecular weight of 175.

Nature of the Semicarbazone of the Reaction Product of Ascorbic Acid and Formaldehyde

The analysis of this compound indicates a mono-derivative with the formula $C_8H_{12}O_5N_3$ and a molecular weight of 207. The prosthetic portion of the molecule would have a formula of $C_5H_9O_5$ and a molecular weight of 148.

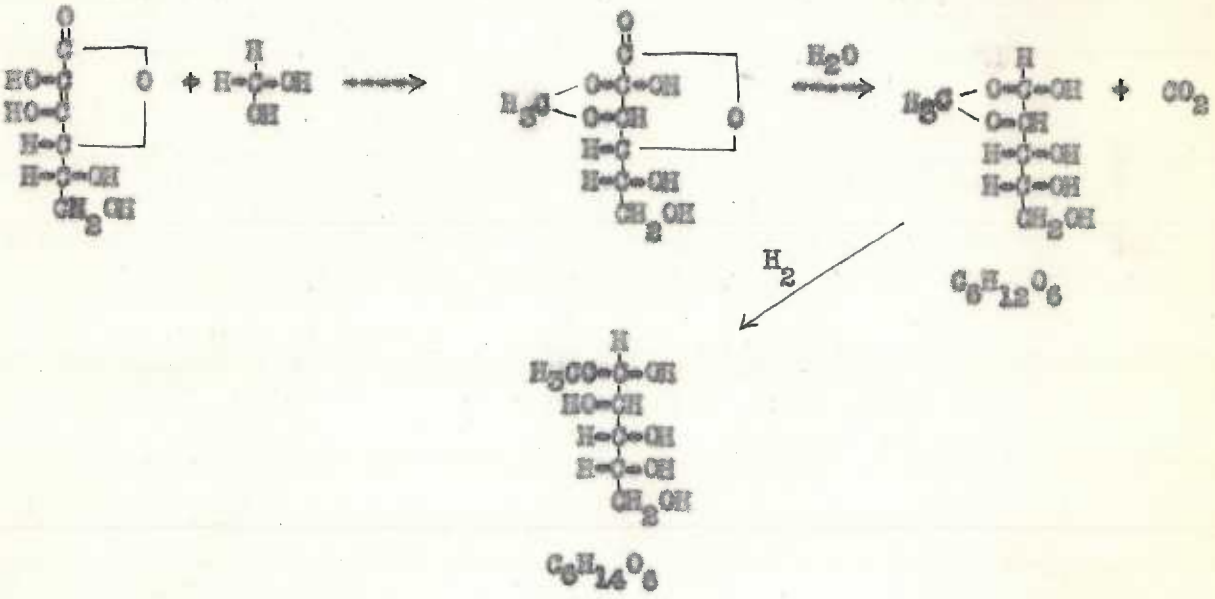
The formula for the semicarbazone may be similar to the following:



Such a compound might be derived from ascorbic acid after decarboxylation. This compound has the formula $C_6H_{13}O_5N_3$, a molecular weight of 207, and contains 20.3% N, 34.8% C, 39.6% O, 6.28% H. This product may have been formed also by primary condensation of formaldehyde.

Possible Mechanisms for the Reaction between Ascorbic Acid and Formaldehyde

From the foregoing evidence it is obvious that ascorbic acid can, and does, react with formaldehyde in more than one way. At least two products of this reaction have been demonstrated and isomerization makes possible many more. Although no definite answer can be given as to how this reaction proceeds, yet certain possibilities can be ruled out.

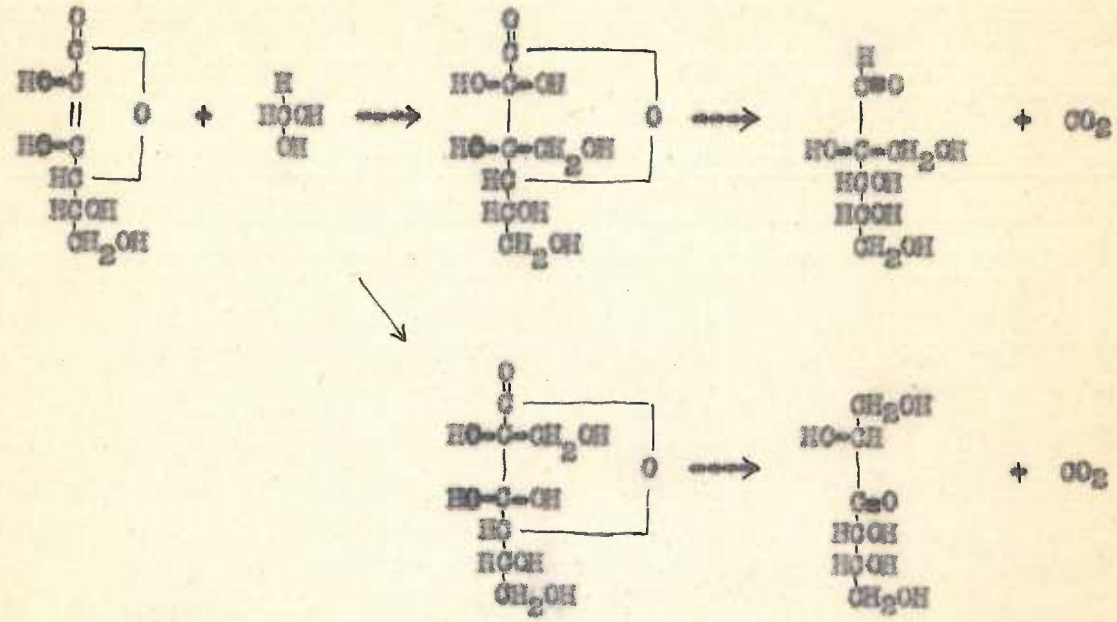


$C_6H_{12}O_6$

$C_6H_{14}O_6$

The above scheme would account for the number of hydroxyl groups found, formation of a methoxyl group on hydrogenation, and blocking of

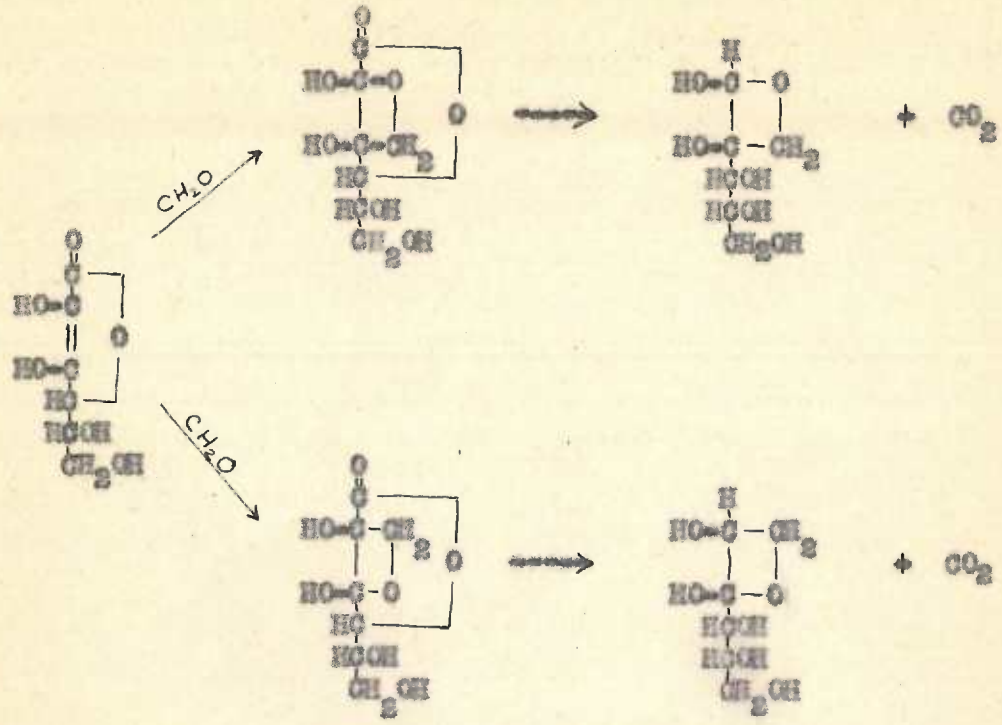
the reducing power with the delayed production of carbon dioxide, but such a methylene ether group could never be demonstrated. Further, no acetal formation occurred.



Nunin found that formaldehyde added to the enol form of benzoin in a manner similar to the reaction above. Neither of the two types of products shown are probable because they contain more hydroxyl groups than the number found, nor could a methoxyl group be formed on hydrogenation.

In the next scheme two important facts are utilized. First, in many reactions formaldehyde acts as if its structure were

Second, it is known that carbon three in ascorbic acid is more negative than carbon two.



Such products as these would possibly account for the finding of both a methyl and a methoxyl group in the hydrogenated product. Although one more hydroxyl group is present than has been found, it will be noticed that one of these is tertiary and as such might be difficult to demonstrate.

It may be seen that none of the schemes above is satisfactory. No explanation can be given for the breaking of the rather stable lactone ring of ascorbic acid in a non-oxidizing medium although Haworth [32] states that etherization of the enediol group increases the tendency for the lactone ring to break. It is also difficult to understand why such a mild reagent as formaldehyde should cause such widespread and irreversible changes in the ascorbic acid molecule. Finally, the possibility that formaldehyde condenses to some extent under these conditions has not been ruled out.

Although the whole problem dealt with here has not been solved it is felt that a groundwork has been laid. Enough facts have been obtained and organized so that at least a partial picture of this hitherto completely unknown process may be visualized. It is hoped that future investigators, guided by this work, will fill in the blanks.

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