THE OXIDATION OF ASCORBIC ACID AS INFLUENCED BY INTESTINAL BACTERIA[1,2]

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INTRODUCTION

Stepp and Schroder (1935), and Stepp (1936), reported that certain strains of intestinal bacteria, particularly "*B. coli communis*" and "*B. paratyphosus B*", were capable of destroying ascorbic acid (vitamin C), while other intestinal bacteria were without action on this substance. They discussed the possibility that some cases of scurvy might be caused by the destruction of ascorbic acid by bacteria in the upper intestinal tract before the acid could be absorbed. Marin (1936) described a clinical case of scurvy which he believed had been caused by an "infection of *B. coli* and *B. paratyphosus B* in the upper intestinal tract."

Prompted by the above work the investigation here reported was carried on to study the action of normal intestinal bacteria on ascorbic acid, and factors influencing such action. In the event that certain kinds of intestinal bacteria may destroy ascorbic acid while others do not, it would be advisable from the standpoint of proper human nutrition to attempt to control these offending organisms in the intestinal tract. Such an alteration of intestinal flora possibly might be brought about by diet. However, this investigation in itself is concerned with an *in vitro* study of certain kinds of intestinal bacteria and their action on ascorbic acid under different conditions.

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[2] Based on portions of a thesis presented by William B. Esselen, Jr., April, 1938, to the Faculty of the Graduate School of the Massachusetts State College in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

REVIEW OF LITERATURE

The literature contains very little information pertaining to bacteria and ascorbic acid. Einhauser (1937) found that patients with achlorhydria were not easily saturated with ascorbic acid administered by mouth, and he attributed this effect to the destructive action of an acid condition and of bacteria in the stomach and upper part of the intestine. This work corroborates that of Stepp and of Marin mentioned above. Gagyi (1936), and Gagyi and Ujsaghy (1936), found that certain bacteria, particularly the more virulent pathogens, possess a capacity for destroying ascorbic acid, and that the inability of these organisms to grow in a 0.2 per cent solution of this substance and their ability to destroy it run parallel. Hou (1936) reported that the destruction of vitamin C experimentally in distilled water or in tap water was caused partly by microorganisms.

The incorporation of ascorbic acid in culture media for the cultivation of anaerobes has been investigated to a limited extent. Ehrismann (1935) found that ascorbic acid in a dilution of 1:1000 favored the growth of obligate anaerobes even under aerobic conditions. Kligler and Guggenheim (1938) found that vitamin C used in culture media for *Clostridium welchii* reduced the oxidation-reduction potential of the medium sufficiently so that this organism would grow even in the presence of air or of oxygen. They also observed that the loss of ascorbic acid was less in a growing culture of *C. welchii* than in sterile control media.

Kligler, Leibowitz, and Berman (1937), in a study of the effect of ascorbic acid on toxin production by *Corynebacterium diphtheriae* in culture media, found that ascorbic acid was rapidly oxidized in culture media at a temperature of 30° to 37°C., but that it remained unoxidized longer in the presence of bacterial growth. They suggested that ascorbic acid may destroy by an oxidation-reduction reaction the toxin produced by this organism, and that the mechanism is similar to that of the toxin-destroying action of aldehydes in general.

Tkachenko (1936) appears to be the only one who has reported

studies on the ability of bacteria to reduce dehydroascorbic acid to ascorbic acid. He found that this conversion took place in cultures of *Lactobacillus bulgaricus*, *L. acidophilus*, and *L. leichmanni*.

Some work has been done relative to the effect of microbial growth on the vitamin C content of foods. Lepkovsky, Hart, Hastings, and Frazier (1925) found that *Streptococcus lactis* had no effect on the vitamin C content of orange and tomato juices. Bifano and Servazzi (1935) infected lemons with *Penicillium digitatum* and found that their vitamin C content was the same as that of non-infected lemons.

Esselen (1938) found that a number of different intestinal bacteria exerted a "protective action" on the oxidation of ascorbic acid. He suggested that this "protective action" might be correlated with bacterial growth activities. Kendall and Chinn (1938) reported an investigation on the decomposition of ascorbic acid by bacteria. As a result of their work it would appear that specific strains of bacteria, rather than bacterial "species" are able to ferment ascorbic acid. They also reported that certain bacteria growing in an ascorbic acid medium exhibit a "protective action," that is, actually retard the oxidation of ascorbic acid. They found, too, that glucose exerts a definite sparing action upon the fermentation of ascorbic acid by bacteria.

Thus, a review of the literature indicates that bacteria may be classified into three groups as regards the stability of ascorbic acid in their presence, namely: (1) those organisms which destroy ascorbic acid; (2) those which protect ascorbic acid from oxidation; and (3) those which have no influence on this substance.

EXPERIMENTAL WORK

Methods

Throughout this investigation cultural methods described below were employed in the study of the action of bacteria on ascorbic acid. The media used were physiological saline (0.85 per cent sodium chloride), and nutrient broth of the following composition:

"Bacto" beef extract	3.0 grams
"Bacto" peptone	5.0 grams
Carbohydrate (when used)	10.0 grams
Distilled water	1000.0 ml.
Reaction	pH 6.3 to 6.5

This medium was prepared and distributed in 50 ml. test tubes containing 30 ml. of medium, or in 250 ml. Erlenmeyer flasks containing 100 ml. of the medium, and autoclaved for 20 minutes at 15 pounds pressure. One-milliliter portions of a 24-hour nutrient broth culture of the organism studied were used for inoculation. An incubation temperature of 37°C. was used at all times. Pure crystalline ascorbic acid was employed, and the desired amount added to the medium in aqueous solution.

The ascorbic acid content of the medium was determined at the beginning and end of each experiment by a modification of the 2,6-dichlorophenolindophenol dye method of Tillmans as described by Bessey and King (1933). The actual method employed throughout this study consisted in placing 20.0 ml. of the culture mixture into a 50 ml. flask and adding 5.0 ml. of glacial acetic acid (sufficient to lower the pH value of the titration mixture below 2.0). The acidified solution was titrated immediately with a standardized solution of the 2,6-dichlorophenolindophenol dye. The titration was completed within two minutes in each case. Determinations were made in duplicate or in triplicate, and good checks were obtained. For convenience, all data are reported in terms of milligrams of ascorbic acid per 20.0 ml. of medium, and as the per cent of loss of ascorbic acid during the incubation period. In studying the effect of bacteria on ascorbic acid different incubation periods were used, and the ascorbic acid content of the mixture was determined at the beginning and at the end of each incubation period. Thus, it was possible to calculate the per cent loss of ascorbic acid as influenced by the various factors studied. Throughout most of this work much smaller concentrations of ascorbic acid have been used than were used by previous workers, because, under actual conditions existing in the intestinal tract, ascorbic acid is present in only relatively small amounts.

From a preliminary study it was found that ascorbic acid is

rapidly oxidized in uninoculated culture media. Consequently, it was decided to use a five-hour incubation period in studying the influence of bacteria on ascorbic acid because, on longer incubation, the ascorbic acid itself would be practically all destroyed without bacterial influence and it would be difficult to interpret the results as to the influence that bacteria might have on this oxidation. It was also found that, throughout a range of pH values from 3.85 to 9.40, the degree of acidity had no significant influence on the rate of oxidation of ascorbic acid under the conditions of the experiment. Thus, it would seem that the change in the reaction of the medium due to bacterial action would not be a factor in influencing the oxidation of ascorbic acid because this substance is rapidly oxidized throughout the normal pH range of bacterial growth.

The oxidation of ascorbic acid in nutrient and in carbohydrate broth inoculated with various intestinal bacteria

As a preliminary step in studying the destruction of ascorbic acid as influenced by bacterial growth, eleven strains of *Escherichia coli*, and one strain each of *Aerobacter aerogenes*, *Salmonella enteritidis*, *Salmonella pullorum*, *Salmonella aertrycke*, *Eberthella typhosa*, *Bacillus subtilis*, and *Proteus vulgaris* were studied with reference to their influence on ascorbic acid in nutrient broth, carbohydrate broth, and physiological saline by the method described above. The carbohydrates employed were glucose, galactose, lactose, maltose, sucrose, dextrin, and starch. The pH value of each culture was determined at the end of the incubation period by means of a Beckman pH meter. The data obtained are presented in table 1.

Ascorbic acid determinations on control cultures of the organisms with no ascorbic acid present gave no indication that ascorbic acid or ascorbic acid-like substances were synthesized by the bacteria. In no instance did the bacteria cause any greater destruction of ascorbic acid than that which occurred in the uninoculated control medium. Most of the organisms, particularly the *Aerobacter* and *Escherichia* strains, exerted a pronounced inhibitory action on the oxidation of ascorbic acid.

TABLE 1

Final pH of the medium and loss of ascorbic acid by oxidation in inoculated nutrient broth, carbohydrate broth, and physiological saline

Incubation five hours at 37°C.

ORGANISM	NUTRIENT BROTH		GLUCOSE BROTH		GALACTOSE BROTH		LACTOSE BROTH		MALTOSE BROTH		SUCROSE BROTH		DEXTRIN BROTH		STARCH BROTH		PHYSIOLOGICAL SALINE, PER CENT LOSS
	pH	Per cent loss	pH	Per cent loss	pH	Per cent loss	pH	Per cent loss	pH	Per cent loss	pH	Per cent loss	pH	Per cent loss	pH	Per cent loss	
Control	6.3	85	5.8	80	5.8	78	4.7	89	5.9	88	5.8	86	5.8	84	6.3	88	47
A. aerogenes (A101)	5.9	51	4.6	37	4.9	65	5.1	71	5.4	67	4.7	71	5.4	54	6.3	69	29
E. coli (Crooks)	5.7	56	4.4	28	5.1	28	5.3	51	5.4	40	5.6	66	5.3	35	6.3	72	31
E. coli (A1)	5.3	64	4.7	28	5.3	31	5.4	65	5.8	54	5.8	66	5.6	40	6.1	72	31
E. coli (A2)	5.7	68	4.7	30	5.2	42	5.8	72	5.8	71	5.8	74	5.7	42	6.3	73	35
E. coli (A3)	5.6	75	4.6	32	5.3	47	5.4	63	5.7	65	5.8	74	5.6	54	6.3	81	25
E. coli (A4)	5.7	67	4.6	18	5.3	42	5.3	71	5.7	52	5.8	74	5.6	48	6.1	71	33
E. coli (A11)	5.5	63	4.6	26	5.2	37	5.5	61	5.8	49	5.8	64	5.7	38	6.3	64	29
E. coli (A12)	5.7	67	4.7	26	5.2	38	5.4	61	5.8	61	5.8	63	5.7	37	6.0	73	27
E. coli (A20)	5.8	49	4.6	31	5.3	34	5.4	60	5.8	56	5.8	61	5.8	37	6.4	63	11
E. coli (A21)	5.5	62	4.8	31	5.6	33	5.4	70	5.8	58	5.8	61	5.8	29	6.2	64	27
E. coli (A25)	5.7	63	4.8	33	5.4	41	5.5	67	5.7	73	5.1	64	5.6	40	6.2	71	
E. coli (A30)	5.3	69	4.7	49	5.2	22	5.5	74	5.7	69	5.0	68	5.5	45	6.0	75	
S. pullorum	5.2	82	5.9	80	6.0	75	6.2	87	6.2	79	6.0	80	6.0	80	6.4	79	
S. aertrycke	5.7	57	4.8	58	5.4	34	6.2	88	6.0	65	6.0	66	5.9	42	6.3	56	
S. enteritidis	5.8	70	4.8	50	5.3	37	6.1	88	5.9	54	6.0	66	5.8	36	6.1	63	47
E. typhosa	6.0	78	5.0	50	5.4	66	6.1	83	5.8	79	5.5	71	5.8	62	6.3	80	38
P. vulgaris	5.9	65	5.8	65	6.0	66	6.2	67	6.2	71	6.1	80	5.9	47	6.3	77	
B. subtilis	5.8	80	6.0	83	6.0	73	6.1	70	6.2	85	6.1	78	6.1	81	6.2	81	42

The cultures of those organisms which grew most rapidly exerted the strongest inhibitory action on the oxidation of ascorbic acid. The presence of a readily fermentable carbohydrate in the medium still further enhanced the ability of the microörganisms to inhibit the oxidation of ascorbic acid. In physiological saline the organisms appeared to exert a slight inhibitory action on the oxidation of ascorbic acid in some cases, but, as with the nutrient broth, in no instance did they cause any destruction of this substance. It is also evident from table 1 that the degree of acidity produced by the bacteria under observation is not a factor in the ability of the bacteria to inhibit the oxidation of ascorbic acid. There appears to be no correlation between the acidity produced by an organism and its ability to inhibit the oxidation of ascorbic acid.

Influence of relative number of bacteria and their stage of growth on the oxidation of ascorbic acid

A test was conducted to determine the influence of the size of inoculum on the loss of ascorbic acid by *Escherichia coli*, *Aerobacter aerogenes*, and *Proteus vulgaris*. From table 2 it may be seen that, in general, as the size of the inoculum was increased the oxidation of ascorbic acid was decreased. These results indicated that the inhibitory effect of bacteria on the oxidation of ascorbic acid increased with the increase in the number of bacteria present. In the event that such is the case, older but still active cultures of bacteria which would contain large numbers of organisms should inhibit the oxidation of ascorbic acid to a greater extent than the relatively young cultures so far studied.

A series of experiments were carried out to test this hypothesis. Ascorbic acid was added to 5, 17, 24, 48, and 72-hour nutrient broth and glucose broth cultures of the organisms previously used. After five hours the loss of ascorbic acid was determined. The data so obtained are presented in table 3. The ability of all of the strains studied to inhibit the oxidation of ascorbic acid increased with the age of the cultures up to 24 hours, but beyond this time the cultures of the organisms appeared slowly to lose this property. The cultures exhibited their most marked inhib-

itory action towards ascorbic acid oxidation when they were in the stages of growth in which the maximum number of viable organisms was present in the medium (Jordan and Falk (1928)). Organisms in the glucose broth developed the power to inhibit the oxidation of ascorbic acid sooner than did duplicate cultures in nutrient broth.

Apparently, then, the actively growing bacteria bring about some change in the media which tends to inhibit the oxidation of

TABLE 2

Influence of the quantity of inoculum on the loss of ascorbic acid in nutrient broth incubated at 37°C. for five hours

ORGANISM	QUANTITY OF INOCULUM	ASCORBIC ACID PER 20 ML. NUTRIENT BROTH	LOSS IN FIVE HOURS
	ml.	*mgm.*	*per cent*
Control (start)	None	0.65	
Control (5 hours)	None	0.07	89
E. coli (Crooks)	0.1	0.07	89
	1.0	0.20	69
	5.0	0.45	30
	10.0	0.35	46
A. aerogenes	0.1	0.10	85
	1.0	0.15	77
	5.0	0.47	28
	10.0	0.60	8
P. vulgaris	0.1	0.07	89
	1.0	0.08	88
	5.0	0.08	88
	10.0	0.08	88

ascorbic acid. Several possibilities present themselves in explanation of what happens during bacterial growth which might inhibit the oxidation of ascorbic acid, namely: (1) the formation of un-ionized copper complexes with protein decomposition products, by which the catalytic power of small amounts of copper naturally present (Dunham (1938)) might be destroyed (Barron, Barron, and Klemperer (1936)); (Ettisch, Sachsse, and Beck (1931)); (2) a change in the oxygen tension of the medium and its saturation with other gases given off as a result of bacterial

TABLE 3

Influence of the age of culture on the rate of oxidation of ascorbic acid in nutrient broth

ORGANISM	AGE OF CULTURE*	PER CENT LOSS OF ASCORBIC ACID IN 5 HOURS AT 37°C.†	
		Nutrient broth	Glucose broth
	hours	*per cent*	*per cent*
A. aerogenes (A101)	0	56	36
	5		8
	17	8	3
	24	0	0
	48	24	
	72	17	
E. coli (Crooks)	0	37	19
	5	36	
	17	19	7
	24	0	0
	48	15	
	72	22	
E. coli (A2)	0	63	11
	5	48	
	17	17	6
	24	0	0
	48	17	
	72	20	
E. coli (A25)	0	67	37
	5	45	
	17	37	3
	24	0	14
	48	22	
	72	31	
P. vulgaris	0	85	80
	5	49	17
	17	11	3
	24	0	0
	48	11	
	72	26	
B. subtilis	0	90	88
	5	47	33
	17	20	0
	24	32	0
	48	23	
	72	69	

* Age of culture when ascorbic acid was added.

† Media contained 0.87 mgm. of ascorbic acid at start of incubation periods.

growth activity; and (3) the production of bacterial metabolic products which might prevent the ascorbic acid from being oxidized.

The influence of the concentration of ascorbic acid on its rate of oxidation and on the growth of bacteria

Several series of flasks each containing 100 ml. of glucose broth were prepared so as to contain ascorbic acid in concentrations approximating 1.0, 0.1, 0.01, and 0.001 per cent respectively. Inoculums of three strains of *Escherichia coli* and one strain each of *Aerobacter aerogenes*, *Proteus vulgaris*, and *Bacillus subtilis* were added separately to the different series of flasks for 18 hours. After incubation, plate counts were made from each flask in order to determine the influence of the concentration of ascorbic acid on the growth of the bacteria. Ascorbic acid determinations were also made so that the per cent of loss of this substance as influenced by its concentration and the presence of bacterial growth in the medium could be calculated. These data are summarized in table 4. From this information it is quite evident that bacterial growth is inhibited as the concentration of ascorbic acid in the medium is increased above certain concentrations, depending upon the kind of bacteria employed. However, with the slower growing organisms such as *Proteus vulgaris*[3] and *Bacillus subtilis*, concentrations of ascorbic acid up to 0.01 per cent and 0.1 per cent respectively appeared to stimulate growth.

At the beginning of these experiments the influence of the concentration of ascorbic acid on the pH values of the media was measured and was found to be as follows:

Glucose broth without ascorbic acid	pH 6.31
Glucose broth plus 0.001 per cent ascorbic acid	pH 6.31
Glucose broth plus 0.01 per cent ascorbic acid	pH 6.08
Glucose broth plus 0.1 per cent ascorbic acid	pH 4.60
Glucose broth plus 1.0 per cent ascorbic acid	pH 3.65

[3] While *Proteus vulgaris* is generally considered to be a rapidly growing organism, the strains employed by the author grew slowly in comparison with *Escherichia coli*.

TABLE 4

The influence of concentration of ascorbic acid upon its rate of oxidation and upon bacterial growth in glucose broth at 37°C.

ORGANISM	ASCORBIC ACID PER 20 ML. MEDIA	LOSS OF ASCORBIC ACID IN 18 HOURS	NUMBER OF ORGANISMS PER ONE ML. OF MEDIA AFTER 18 HOURS
	mgm.	*per cent*	
Control (start)	181.93		
	18.13		
	1.75		
	0.11		
Control (18 hours)	169.93	7	
	7.93	56	
	0.08	95	
	0.01	00	
A. aerogenes (A101)	181.90	0	10,500
	15.93	12	100,000,000
	1.37	22	100,000,000
	0.11	0	130,000,000
	None		130,000,000
E. coli (Crooks)	139.93	23	13,500
	17.73	2	23,400,000
	1.61	8	80,000,000
	0.09	18	170,000,000
	None		250,000,000
E. coli (A2)	181.00	0	30,000
	15.33	14	14,400,000
	0.93	47	200,000,000
	0.05	55	132,000,000
	None		250,000,000
E. coli (A25)	179.93	1	20,000
	16.73	8	12,000,000
	0.79	55	50,000,000
	0.03	73	86,000,000
	None		100,000,000
P. vulgaris	141.93	22	20,000
	13.13	28	7,800,000
	0.54	69	70,000,000
	0.02	82	128,000,000
	None		20,000,000
B. subtilis	169.93	7	1,600
	18.10	0	4,200,000
	0.27	85	43,000,000
	0.01	99	150,000,000
	None		800,000

The above information would suggest that the inhibition of bacterial growth observed as a result of increasing the concentration of ascorbic acid may be due to the increased hydrogen ion concentration of the media produced by the ascorbic acid.

The production of carbon dioxide by bacteria as a factor influencing the stability of ascorbic acid

In the event that the observed inhibitory action of bacteria on the oxidation of ascorbic acid as reported above might have been due to gases produced by the organisms, the influence of carbon dioxide and of hydrogen on ascorbic acid oxidation was studied. Eighteen different bacterial strains were inoculated into flasks of nutrient broth to which ascorbic acid was added according to the technique previously employed. The flasks were incubated in anaerobe jars for five hours at 37°C. in atmospheres of carbon dioxide and of hydrogen respectively. The results are presented in table 5. In comparing these results with those in table 1 it may be seen that, in general, the oxidation of ascorbic acid in an atmosphere of hydrogen is similar to that which takes place in nutrient broth under aerobic conditions. The carbon dioxide exerted an inhibiting effect on the oxidation of ascorbic acid to a degree parallel to that exerted by the bacteria when they were cultured in nutrient broth containing a readily fermentable carbohydrate. Thus, it would seem that the protective action of the bacteria on ascorbic acid may be due, at least in part, to the carbon dioxide which they produce. This possibility was investigated by measuring the quantities of total dissolved gas and of dissolved carbon dioxide which were present in the cultures as a result of bacterial metabolism. The manometric method of Van Slyke and Neill, as described by Hawk and Bergeim (1931) was employed for the measurement. The quantities of total gas and of carbon dioxide produced by the bacteria in the cultures were compared with the degree of oxidation of ascorbic acid in the medium as influenced by the bacteria present.

The results are given in table 6, from which it may be seen that the organisms which significantly inhibited the oxidation of

ascorbic acid also produced considerable carbon dioxide and total gas in comparison with those which did not retard the oxidation of ascorbic acid. Although these data show a correlation between carbon dioxide production by bacteria and their ability to inhibit

TABLE 5

Influence of atmospheres of carbon dioxide and hydrogen on the oxidation of ascorbic acid in nutrient broth inoculated with different intestinal bacteria in five hours at 37°C.

ORGANISM	IN ATMOSPHERE OF HYDROGEN		IN ATMOSPHERE OF CARBON DIOXIDE	
	Ascorbic acid per 20 ml. broth	Loss	Ascorbic acid per 20 ml. broth	Loss
	mgm.	*per cent*	*mgm.*	*per cent*
Control (start)	0.74		0.72	
Control (5 hours)	0.13	82	0.40	44
A. aerogenes (A101)	0.24	68	0.47	35
E. coli (Crooks)	0.32	57	0.42	42
E. coli (A1)	0.43	42	0.52	28
E. coli (A2)	0.30	59	0.53	26
E. coli (A3)	0.34	54	0.55	24
E. coli (A4)	0.32	57	0.60	17
E. coli (A11)	0.36	51	0.50	30
E. coli (A12)	0.31	58	0.55	24
E. coli (A20)	0.33	55	0.50	30
E. coli (A21)	0.35	53	0.50	30
E. coli (A25)	0.39	47		
E. coli (A30)	0.41	45		
S. pullorum	0.18	76		
S. aertrycke	0.33	55	0.40	44
S. enteritidis	0.32	57	0.60	17
E. typhosa	0.29	61		
P. vulgaris	0.19	74	0.50	30
B. subtilis	0.16	78	0.40	44

the oxidation of ascorbic acid, the evidence does not prove that the carbon dioxide is the only inhibiting factor involved.

In order to determine the rôle, if any, of carbon dioxide production by bacteria in the prevention of the oxidation of ascorbic

TABLE 6

The total amounts of dissolved gas and of carbon dioxide produced by bacteria in glucose broth in five hours at 37°C. and the oxidation of ascorbic acid under the same conditions

ORGANISM	ASCORBIC ACID PER 20 ML. OF BROTH	LOSS	TOTAL VOL. OF GAS AS CC. PER 100 ML. OF BROTH	VOL. OF CARBON DIOXIDE PER 100 ML. OF BROTH
	mgm.	*per cent*		
Control (start)	0.75			
Control (5 hours)	0.10	86	2.1	0.0
A. aerogenes (A101)	0.46	39	12.8	4.7
E. coli (Crooks)	0.52	30	6.2	1.9
E. coli (A1)	0.55	26	8.8	3.4
E. coli (A2)	0.39	48	7.7	2.2
E. coli (A3)	0.45	37	9.0	1.9
E. coli (A4)	0.41	43	8.0	1.9
E. coli (A11)	0.47	35	9.6	2.3
E. coli (A12)	0.45	37	9.3	3.2
E. coli (A20)	0.49	29	8.8	2.1
E. coli (A21)	0.47	35	9.2	2.8
E. coli (A25)	0.43	42	8.8	2.3
E. coli (A30)	0.47	35	8.3	2.1
S. pullorum	0.12	84	2.5	0.0
S. aertrycke	0.37	50	8.2	1.8
S. enteritidis	0.40	46	9.3	1.9
E. typhosa	0.20	73	3.2	0.0
P. vulgaris	0.19	75	2.4	0.0
B. subtilis	0.11	85	2.0	0.0

TABLE 7

The influence of dissolved carbon dioxide on the rate of oxidation of ascorbic acid in uninoculated glucose broth in five hours at 37°C.

GROUP	DISSOLVED CARBON DIOXIDE PER 100 ML. BROTH	ASCORBIC ACID PER 20 ML. OF BROTH	LOSS
	cc.	*mgm.*	*per cent*
Control (start)	0.0	0.77	
Control (5 hours)	0.0	0.08	90
Series A, plus CO_2	2.0	0.19	75
Series B, plus CO_2	4.6	0.30	61
Series C, plus CO_2	13.4	0.30	61

acid, an experiment similar to the last one was conducted, only in this case three series of an uninoculated medium containing

graduated amounts of dissolved carbon dioxide were used. Ascorbic acid was added to each flask and carbon dioxide dissolved in the medium by placing the flasks in anaerobe jars and adding carbon dioxide to the atmosphere of the jars. When the medium contained the desired amount of this gas (previously determined) the anaerobe jars containing the flasks of medium were incubated for five hours at 37°C. At the end of this period the ascorbic acid and carbon dioxide contents of each series were determined. The data presented in table 7 show that the presence of carbon dioxide in glucose broth did exert a small inhibitory effect on the rate of oxidation of ascorbic acid. However, this inhibitory effect was not as marked as that produced by bacteria which evolve carbon dioxide when grown in glucose broth. The uninoculated glucose broth used in the last experiment contained a greater concentration of dissolved carbon dioxide than was produced by bacteria under similar conditions. This fact indicated that carbon dioxide production by bacteria is not the only factor in the mechanism whereby they inhibit the oxidation of ascorbic acid.

Assuming that the carbon dioxide production by bacteria was one factor influencing their inhibitory action on the oxidation of ascorbic acid, another set of experiments was conducted in order to find out whether bacteria exert this effect by combinations of three factors, namely: (1) carbon dioxide production, (2) removal of oxygen from the medium, and (3) the formation of un-ionized copper complexes. In this experiment uninoculated glucose broth was used as the medium, and the flasks were incubated in anaerobe jars for five hours at 37°C. The influence of carbon dioxide was determined by adding the gas at 20 pounds pressure to the atmosphere in the anaerobe jars. The effect of the removal of oxygen from the medium by bacterial action was simulated by evacuating the anaerobe jars with a vacuum pump; and the formation of un-ionized copper complexes by the bacteria was duplicated by adding 0.03 gram of 8-hydroxyquinoline to each 100 ml. of medium. (It has been previously shown by Barron, Barron, and Klemperer (1936) that 8-hydroxyquinoline destroys

the catalytic action of small amounts of copper on the oxidation of ascorbic acid.) The results are presented in table 8.

Each of the above factors exerted a certain amount of inhibitory action on the oxidation of ascorbic acid, but when they were used in combinations of two together, or of all three together, their combined inhibitory effect was greater than the total inhibitory effect of these three factors, namely, carbon dioxide, vacuum, and 8-hydroxyquinoline, employed separately. In

TABLE 8

The inhibitory effect of carbon dioxide, vacuum, and 8-hydroxyquinoline on the oxidation of ascorbic acid in uninoculated glucose broth in 5 hours at 37°C.

EXPERIMENTAL GROUP	ASCORBIC ACID PER 20 ML. OF BROTH	LOSS	INHIBITION OF ASCORBIC ACID OXIDATION*
	mgm.	*per cent*	*per cent*
Control (start)	0.79		
Control (5 hours)	0.10	87	
Control plus 8-hydroxyquinoline	0.12	85	2
Control plus vacuum	0.19	76	11
Control plus carbon dioxide	0.24	70	17
Control plus 8-hydroxyquinoline plus vacuum	0.32	60	27
Control plus 8-hydroxyquinoline plus carbon dioxide	0.47	40	47
Control plus vacuum plus carbon dioxide	0.47	40	47
Control plus vacuum plus carbon dioxide plus 8-hydroxyquinoline	0.69	13	74

* Note: The per cent inhibition of ascorbic acid oxidation refers to the difference between the per cent loss of ascorbic acid in the control medium and that in the control media plus the substance under consideration.

comparing these data with those previously obtained with bacteria it would seem that the ability of the various bacteria to inhibit the oxidation of ascorbic acid might be due to their ability to form un-ionized copper complexes, and to their production of carbon dioxide in, and their removal of oxygen from, the medium. It is not unreasonable to suspect that the intensity of the action of the above three mechanisms would vary with different species of bacteria and with their stage of growth. When studied separately the inhibitory action of carbon dioxide was greater

than that of either a vacuum or of 8-hydroxyquinoline. The 8-hydroxyquinoline was the least active in this respect, but when this substance was used in combination with carbon dioxide or with a vacuum the resultant inhibitory action was more pronounced.

If it is assumed that all bacteria in their growth in culture media are capable of forming un-ionized copper complexes, then bacterial species which produce carbon dioxide in their metabolic processes should exert a more marked inhibitory effect on the oxidation of ascorbic acid than those organisms which do not produce carbon dioxide and, also, the inhibitory effect of carbon dioxide-producing species should be greater than that of carbon dioxide alone when added to uninoculated culture media. Likewise, those species producing little or no carbon dioxide should exert a stronger inhibitory action when grown in an atmosphere of this gas. Also, species such as *Escherichia coli*, when they are grown in nutrient broth to which carbon dioxide is added, should exert an inhibitory effect similar to that which they exert when grown in glucose broth. The reduced oxygen tension of the medium in which the bacteria are grown, which was duplicated by the use of a vacuum, is another factor which should influence the ability of bacteria to inhibit the oxidation of ascorbic acid. However, this factor is likely to be variable due to the cultural conditions and the changing vigor of the bacterial growth.

If the data in tables 1, 5, 6, and 8 are compared it will be seen that the observations made in this investigation substantiate the above explanation of the mechanism by which bacteria inhibit the oxidation of ascorbic acid.

The influence of killed cultures of bacteria and of cell-free culture filtrates on the oxidation of ascorbic acid

It has been suggested that the inhibitory action of bacteria on the oxidation of ascorbic acid is due principally to the production of carbon dioxide and the reduced oxygen tension of the medium as a result of their metabolic activities. If such is the case it would seem that killed cultures of the bacteria, or the cell-free filtrates from such cultures, would not exhibit the inhibitory

action observed with actively growing cultures of bacteria. Three series of tests were conducted to investigate this hypothesis.

Twenty-four-hour cultures of *Escherichia coli* and of *Salmonella aertrycke* in glucose broth were filtered through Berkefeld filters. Ascorbic acid was added to these culture filtrates and they were incubated. At the end of five hours the per cent of loss of ascorbic acid was determined. Also, twenty-four-hour cultures of *Aerobacter aerogenes*, *Escherichia coli*, *Proteus vulgaris*, and *Bacillus subtilis* were killed by 15 pounds steam pressure for 15 minutes, and by the addition of 4.0 ml. of 20 per cent phenol per culture, respectively. Ascorbic acid was added to the killed cultures, and at the end of a five-hour incubation period at 37°C. the per cent of loss of ascorbic acid was determined. In all cases it was found that the absence of actively growing bacteria, whether they were removed from the medium by filtration or were killed, resulted in the loss of the ability of the cultures to inhibit the oxidation of ascorbic acid significantly. Thus, further evidence is provided to show that in order to prevent the oxidation of ascorbic acid in culture media actively growing bacteria must be present.

The ability of bacteria to reduce dehydroascorbic acid

The only report which has been found in the literature pertaining to the reduction of dehydroascorbic acid by bacteria is that of Tkachenko (1936) who reported that such a reduction did take place in cultures of several species of "Lactobacilli." Dehydroascorbic acid was prepared according to the method of Kohman and Sanborn (1937) by oxidizing ascorbic acid in aqueous solution with iodine. Eleven strains of *Escherichia coli*, and one strain each of *Aerobacter aerogenes*, *Salmonella pullorum*, *Salmonella aertrycke*, and *Salmonella enteritidis* were studied with reference to their ability to reduce dehydroascorbic acid. The acid was added to 24-hour cultures of these organisms in glucose broth. At the beginning of the experiment the ascorbic acid in the medium was all present in the dehydro-form, but at the end of five hours two of the strains of *Escherichia coli* had reduced approximately 90 per cent of the dehydroascorbic acid to its

equivalent of ascorbic acid, as may be seen in table 9. Of the organisms studied only these two strains of *Escherichia coli* were capable of bringing about this reduction. Repeated tests furnished definite proof that the two strains of *Escherichia coli* were unique in this respect among the strains employed.

TABLE 9

The reduction of dehydroascorbic acid to ascorbic acid by 24-hour glucose broth cultures of bacteria in five hours at 37°C.

ORGANISM	ASCORBIC ACID PER 20 ML. OF BROTH	REDUCTION OF DEHYDROASCORBIC ACID TO ASCORBIC ACID
	mgm.	*per cent*
Control (start)	0.62*	
Control (5 hours)	0.00	0
A. aerogenes (A101)	0.04	6
E. coli (Crooks)	0.54	87
E. coli (A1)	0.58	94
E. coli (A2)	0.01	2
E. coli (A3)	0.01	2
E. coli (A4)	0.01	2
E. coli (A11)	0.01	2
E. coli (A12)	0.01	2
E. coli (A20)	0.01	2
E. coli (A21)	0.01	2
E. coli (A25)	0.01	2
E. coli (A30)	0.01	2
S. pullorum	0.01	2
S. aertrycke	0.01	2
S. enteritidis	0.01	2

* Ascorbic acid equivalent of dehydroascorbic acid.

SUMMARY

1. It has been found that certain bacteria, particularly members of the coliform group, inhibited the oxidation of ascorbic acid in culture media. The stronger inhibitory action was observed with the more actively growing cultures in which the largest numbers of bacteria were present.

2. Ascorbic acid was rapidly oxidized in uninoculated nutrient broth at 37°C., and this oxidation was retarded by the presence of carbon dioxide, by 8-hydroxyquinoline, and in the absence of oxygen.

3. It is suggested that growing bacteria inhibit the oxidation of ascorbic acid by certain combinations of three factors, namely: (1) the formation of un-ionized copper complexes whereby the catalytic action of the copper is destroyed, (2) the production of carbon dioxide with the subsequent saturation of the medium with it, and (3) the lowering of the oxygen tension of the medium. The most effective inhibitory action was obtained by a combination of all three of these factors together.

4. Two strains only of *Escherichia coli* were able to reduce dehydroascorbic acid to ascorbic acid, while the other bacteria studied did not appear to possess this ability.

5. The inhibitory action of bacteria on the oxidation of ascorbic acid was not observed in relatively large concentrations of this substance because the acidity of the medium was lowered below the point where effective bacterial growth could take place.

6. Killed bacterial cultures, or cell-free filtrates from living bacterial cultures, did not possess the inhibitory action on the oxidation of ascorbic acid that was observed with actively growing bacterial cultures.

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