DECOMPOSITION OF VITAMIN C BY BACTERIA[1]

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The decomposition of vitamin C by bacteria is of double interest to the bacteriologist. Preliminary *in vitro* studies by Young and James (1942) showed that enteric bacteria of the colon-aerogenes group destroy ascorbic acid with great facility; these findings suggest that, in spite of a diet adequate in this substance, symptoms of vitamin C deficiency in some persons may be traced to an intestinal flora high in ascorbic-acid-fermenting bacteria. Einhauser (1936), Hetenyi (1935) and Marin (1936) offer the suggestion that the failure of response to a diet rich in vitamin C, and the positive effects of intramuscular and intravenous injections of this substance, indicate bacterial destruction of ascorbic acid in the gastro-intestinal tract before absorption can take place.

The extreme importance of vitamin C in human physiology and the significance of loss of this substance to the body, regardless of the manner of destruction, are becoming increasingly evident as the result of war research and conditions arising naturally or unavoidably among the fighting forces. Holmes (1942) has indicated that vitamin C may be destroyed by infection, by a number of industrial poisons of a military nature, especially materials used in the manufacture of T.N.T., and that appreciable quantities are lost in heavy perspiration. He reviews the beneficial effects of vitamin C therapy in various types of shock, including heat, traumatic, post-operative and allergic shock. Vitamin C appears to play a significant role in the healing of fractures and of wounds in general. Results reported by Holmes indicate that administration of the vitamin is very effective in the treatment of poisoning due to benzene, toluene, lead, zinc, and basic materials used in the manufacture of T.N.T. and other explosives. Detoxification followed adequate therapy, and there was a repairing action of the poisoned blood formation centers and other affected tissue. Thus, it appears that in numerous common pathological conditions the function of vitamin C extends far beyond the role which it plays in normal metabolism, and that the amount required under these conditions greatly exceeds that needed daily by normal human adults, which according to King and Menten (1935) is only 5 milligrams.

Apart from the influence on human nutrition, the relation of microbic destruction of ascorbic acid to bacterial metabolism is also of considerable interest. Kendall and Chinn (1938) suggested that the vitamin is used by some bacterial species as a carbon food source. The results of preliminary work by Young and James (1942) indicate that the vitamin is utilized in growth as an energy source by certain enteric bacteria. The decomposition was found to take place under

[1] This paper covers in part the dissertation submitted to the Graduate School of Yale University by the senior author as part requirement for the degree of Doctor of Philosophy.

anaerobic as well as aerobic conditions; and, since cell-free filtrates had no effect on the substance, the activation was believed to be associated with the endoenzyme system of the living bacterial cell. The rate of decomposition was found to be most rapid when cells were most viable, pointing to involvement of the respiratory mechanism of the bacterial cell. The presence in the medium of an excess of fermentable substance like glucose delayed or prevented the decomposition, suggesting that in the absence of a readily available carbohydrate, like glucose, the vitamin is used as a substitute for the same purpose.

Because of the implied bearing of vitamin C destruction by bacteria on human nutrition, and because of the importance of the interrelationship between bacterial decomposition of ascorbic acid and bacterial metabolism, the present research was undertaken, with the following objectives in mind:

Making a general survey of the extent of the vitamin-C-decomposing ability of members of various genera in the order *Eubacteriales*.

Determination of the influence of various carbohydrates on the decomposition.

Study of the relation between bacterial growth and the stability of ascorbic acid in synthetic and nutrient broth mediums.

An inquiry into the mode of decomposition of vitamin C by bacteria.

METHODS EMPLOYED

Ascorbic acid nutrient broth was employed in determining the vitamin-C-decomposing ability of various bacterial species. The broth was prepared as follows: a weighed amount of crystalline ascorbic acid was dissolved in a small amount of water, filtered through a Berkefeld filter and the pH adjusted to about 7.0 with a concentrated sterile solution of K_2HPO_4. The filtrate was added to sterile nutrient broth, giving a final concentration of 0.50–0.55 mg. per ml. of broth.

When carbohydrates were used in the medium they were present in a final concentration of 1 per cent. In some of the studies, synthetic ascorbic acid broth and other modifications of the culture medium were employed; these are described later.

After removing an aliquot portion for immediate analysis, the ascorbic acid nutrient broth was distributed in 20 ml. portions in sterile 7 x $\frac{7}{8}$ inch colorimeter tubes, which were then inoculated with the various organisms studied. All cultures and an uninoculated control were incubated under anaerobic conditions at 37°C. Titrations were made frequently, using a modified procedure of Tillman's sodium 2,6-dichlorophenolindophenol method adapted to the Evelyn photoelectric colorimeter. Duplicate portions of 1 ml. of a 1/10 dilution of the culture and of the control were added to 4 ml. of meta-phosphoric-acid buffer solution,[2] thus providing an optimum H-ion concentration of approximately pH 3.5 for the test

[2] *Preparation of meta-phosphoric-acid buffer solution.* A stock solution of 6% metaphosphoric acid was prepared from sticks of glacial metaphosphoric acid. The working reagent consisted of a 3% solution of the acid adjusted to pH 3.5 with citric acid-NaOH buffer solution, as described by Bessey (1938). The buffer was prepared by adding 210 gm. of citric acid to 2,000 ml. of NaOH, and increasing the volume to 2,500 ml. with distilled water. It was found that about 25 ml. buffer were required to adjust 100 ml. of the 3% HPO_3 to pH 3.5.

medium. Then 5 ml. of the indophenol dye solution[3] were added and the color intensity measured with the photoelectric colorimeter. By noting the units of galvanometer deflection and referring the reading to that on a standard reference curve plotted against increasing acid-buffer dilutions of a carefully weighed amount of ascorbic acid, the concentration of vitamin C in the test samples was determined. Under the experimental conditions employed the reaction was immediate; the end point was stable, and the readings could be taken rapidly.

The photoelectric colorimeter was also used to detect growth increase. The effect of the vitamin upon growth was noted by comparing the galvanometer deflection due to turbidity resulting from bacterial growth in ascorbic acid medium with that occurring in broth lacking the vitamin. Since colorimeter tubes were used for all of the cultures, the culture tubes were merely placed in the apparatus after thorough shaking to insure uniform turbidity, and the galvanometer deflection readings referred to standard growth curves in which deflection values were plotted against plate counts of various bacterial species studied.

The anaerobic procedure employed in this investigation consisted in evacuating anaerobic jars and replacing the air with about 10 per cent carbon dioxide and 85–90 per cent hydrogen gas. The culture tubes were placed in the jars, together with a small amount of heated palladinized asbestos, which served as catalyst to effect combination of residual oxygen with hydrogen in the evacuated jar. Anaerobic conditions were employed for the following reasons: (1) because of the importance attached to gastro-intestinal destruction of vitamin C, where the conditions would be anaerobic; (2) because auto-oxidation of vitamin C is retarded greatly under anaerobic conditions; and (3) because, if the decomposition is carried out in the absence of molecular oxygen, implying the operation of a dehydrogenase system, some information is given as to the nature of the decomposition process by bacteria.

GENERAL SURVEY OF THE ABILITY OF VARIOUS SPECIES OF BACTERIA TO DECOMPOSE VITAMIN C

Perhaps the most outstanding observation is the frequency with which the vitamin-C-decomposing property occurs among members of the enteric group of bacteria. As shown in table 1, members of the following genera oxidize ascorbic acid: Escherichia, Aerobacter, Salmonella, Eberthella, Streptococcus (enterococci), Streptococcus (hemolytic), Encapsulatus, Vibrio. Barring two exceptions, it was observed that when one species in a genus possessed this property, it was common to all other genus members tested. *Proteus morgani* actively destroyed ascorbic acid, whereas other species of *Proteus* lacked this ability; however, in many of its characteristics *Proteus morgani* appears to be related more to

[3] *Preparation of indophenol-dye solution.* About 0.1 gm. of the dye powder, sodium 2,6-dichloro-benzenoneindophenol, was added to a liter of warm distilled water, mixed well and filtered. The concentration of dye was so adjusted by adding more water that when 5 ml. of the solution were added to 5 ml. of meta-phosphoric-acid-buffer a galvanometer deflection of exactly 80 units was obtained. Increasing amounts of ascorbic acid decrease the dye color intensity and so decrease the galvanometer deflection. The solution was kept in the refrigerator and remained stable several days.

TABLE 1

Decomposition of ascorbic acid by various bacteria

ORGANISMS INCUBATED UNDER ANAEROBIC CONDITIONS AT 37°C. IN ASCORBIC ACID NUTRIENT BROTH BUFFERED AT pH 7.0	STRAINS OR SPECIES	ASCORBIC ACID OXIDATION*	GAS PROD.†	RECOVERY WITH H_2S‡	REDUCTION OF NILE BLUE§
Escherichia coli	28 strains	+	+	−	+
Aerobacter aerogenes	12	+	+	−	+
Aerobacter cloacae	1	+	+	−	+
Strep. liquefaciens	2	+	−	−	+
Strep. zymogenes	2	+	−	−	
Strep. agalactiae	2	+	−	−	
Strep. fecalis	15	+	−	−	+
Strep. pyogenes	1	+	−	−	
Encapsulatus pneumoniae	1	+	+	−	
Vibrio cholerae (at pH 7.8)	1	+	−	−	
Vibrio metchnikovi (pH 7.8)	1	+	−	−	
Eberthella typhi	1	+	−	−	
Proteus morgani	2	+	+	−	+
Salmonella paratyphi	2	+	+	−	
Salmonella schottmülleri	2	+	+	−	
Salmonella enteritidis	2	+	+	−	+
Salmonella aertrycke	3	+	+	−	+
Salmonella suipestifer	2	+	+	−	
Salmonella anatum	1	+	+	−	+
Salmonella gallinarum	10	+	−	−	+
Salmonella pullorum	33	−			+
Proteus vulgaris	3	−			+
Shigella dysenteriae	Flexner	−			
Shigella dysenteriae	Shiga	−			
Staphylococcus aureus	2	−			
Staphylococcus albus	1	−			
Brucella	3 species	−			
Pasteurella leptiseptica	1	−			
Serratia marcescens	4	−			+
Pseudomonas aeruginosa	2	−			
Flavobacterium vitaminosus	1	−			
Alcaligenes viscosum	2	−			−
Alcaligenes fecalis	2	−			−
Incubated under aerobic conditions					
Pseudomonas fluorescens	2	−			
Bacillus	7 species	−			+

+, complete decomposition in 20–48 hours, as shown by periodic quantitative analysis. Initial amount 0.55 mg. per ml. −, no action after 7–10 days.

* Growth of all cultures listed was moderate to luxuriant in ascorbic acid nutrient broth.

† +, production of gas. Quantitative tests showed a positive correlation between increase in gas production and decrease in ascorbic acid content of the medium. −, no gas production.

‡ −, no recovery of ascorbic acid, and hence irreversible oxidation.

§ +, complete reduction of nile blue under anaerobic conditions by ascorbic acid substrate in the presence of "resting cells." −, no reduction.

the colon-aerogenes group and the salmonellas than to the *Proteus* genus. *Salmonella pullorum* alone, of the various species of *Salmonella* tested failed to de-

compose ascorbic acid; but this organism is relatively asaccharolytic as compared with other members of the group.

Furthermore, with only a few exceptions, not indicated in table 1, it was found that when a given strain fermented the vitamin all strains of the same species also destroyed it. The exceptions were invariably variant strains which showed atypical reactions in other biochemical tests, or failed to grow in the ascorbic acid broth.

Aerobic spore-formers, a few intestinal and many non-intestinal forms did not attack the vitamin in nutrient broth, even though good growth was evident. Members of the following genera failed to act on the substance: Bacillus, Alcaligenes, Pseudomonas, Proteus, Shigella, Staphylococcus, Brucella, Pasteurella, Serratia, Flavobacterium, Chromobacter, Achromobacter.

Vitamin-C-decomposing bacteria appear to possess initial capacity to destroy ascorbic acid, no training or acclimatization technique being necessary. Another outstanding observation is the "all or none" character of the reaction. In every instance where an organism attacked the vitamin at all the decomposition was complete; that is, all of the vitamin in the test medium was destroyed or utilized. With most cultures the destruction was rapid, occurring within 12–22 hours.

Some organisms, especially members of the *Serratia*, *Flavobacterium* and *Pseudomonas* genera, showed marked acceleration of growth in the presence of vitamin C in nutrient broth; yet, these organisms were unable to attack or decompose the substance. The "growth factor" effect may have been due to a favorable influence of a lowered potential of the medium, ascorbic acid being a strong reducing substance.

INFLUENCE OF VARIOUS CARBOHYDRATES ON THE DECOMPOSITION

In earlier studies it was observed that the presence of either glucose or lactose in ascorbic acid nutrient broth deflected attack upon the vitamin by members of the colon-aerogenes group under both aerobic and anaerobic conditions. When the concentration of glucose or lactose was varied, the vitamin disappeared rapidly from the medium, following complete utilization of these sugars.

The present investigation sought to determine the extent to which the vitamin-sparing action occurs among the various groups of organisms that ferment the substance; and to correlate the sparing effect of different carbohydrates with the ability of the bacteria to ferment them. The list of carbohydrates included glucose, lactose, xylose, sucrose, dextrin and mannitol. They were added in 1 per cent concentration to 1 per cent peptone broth containing 0.50 mg. ascorbic acid per ml. buffered at pH 7.0. Several bacterial species listed in table 2 were selected from the large group of vitamin-C-decomposing bacteria to serve as control organisms. Since the principles underlying the results obtained with glucose and lactose apply also to those obtained with xylose, sucrose, dextrin and mannitol, the data for glucose and lactose broth only are presented in table 2.

From the results obtained it may be postulated that when an excess amount of carbohydrate is fermented rapidly by vitamin-C-decomposing bacteria, with acid or acid and gas production, ascorbic acid, when added to the same medium, will

as a rule be spared from oxidation. When the carbohydrate was not fermented at all or attacked slowly by a given species the vitamin was rapidly decomposed, without exception. As indicated in table 2, one strain of *Aerobacter aerogenes* fermented lactose rapidly, protecting the vitamin completely, whereas two other strains of the same species were slow lactose fermenters, and attacked ascorbic acid more readily, completely destroying the substance. Since all of the *Salmonella* species fermented glucose rapidly, the glucose prevented oxidation of ascorbic acid in every instance. On the other hand, since the *Salmonella* species do not attack lactose, this substance had no influence on the total decomposition of vitamin C by these species. The usual period for total oxidation of ascorbic acid was around 15–22 hours. When another substrate present with the vitamin was fermented within this time the vitamin usually was spared completely from oxidation. When a given carbohydrate spared the vitamin from oxidation in a

TABLE 2

Influence of glucose and lactose on the bacterial decomposition of vitamin C

ORGANISMS INCUBATED UNDER ANAEROBIC CONDITIONS AT 37°C. IN BROTH BUFFERED AT pH 7.0	GLUCOSE BROTH (1 PER CENT)			LACTOSE BROTH (1 PER CENT)				
	Plus vitamin C		Ferm. tube	Plus vitamin C		Ferm. tube		
	42 hours	168 hours	18 hours	42 hours	168 hours	18 hours	30 hours	78 hours
E. coli (Ce)	0.42*	0.33	AG†	0.46*	0.46	AG†		
E. coli (Dd)	0.38	0.33	AG	0.47	0.47	AG		
E. coli (Co-3)	0.47	0.44	AG	0.46	0.45	A	AG	
A. aerogenes (5)	0.47	0.45	AG	0.47	0.43	AG		
A. aerogenes (11)	0.40	0.25	AG	0.00		0	0	AG
A. aerogenes (14)	0.47	0.35	AG	0.00		0	0	AG
Strep. fecalis (K)	0.42	0.38	A	0.39	0.32	A	A	A
Strep. fecalis (U)	0.42	0.35	A	0.40	0.30	A	A	A
Strep. fecalis (P)	0.42	0.37	A	0.43	0.38	A	A	A
Strep. liquefaciens	0.41	0.37	A	0.00		0	A	A
Sal. para B (18)	0.42	0.37	AG	0.00		0	0	0
Sal. para B (19)	0.42	0.40	AG	0.00		0	0	0
Sal. enteritidis	0.42	0.41	AG	0.00		0	0	0
Sal. aertrycke	0.42	0.38	AG	0.00		0	0	0
Sal. anatum	0.42	0.39	AG	0.00		0	0	0
Sal. gallinarum	0.42	0.26	A	0.00		0	0	0
Proteus morgani	0.43	0.43	AG	0.00		0	0	0
Uninoculated control	0.33	0.28		0.33	0.28			

* Milligrams ascorbic acid per ml. broth. Initial amount: 0.50 mg. per ml.

† AG indicates both acid and gas production in ordinary glucose and lactose fermentation broths containing no ascorbic acid. 0 indicates no action on the glucose or lactose sugar.

bacterial culture the protection offered was, as a rule, 100 per cent; whereas, if there was no protection the decomposition brought about by bacteria also was 100 per cent.

These studies demonstrate a rather familiar observation in bacterial metabolism. Some bacteria seem to have a preference for certain substrates when two or more fermentable substances are present in the same medium; one or more of the substances may be spared entirely from attack, while the others are readily utilized.

A study of the data in table 2 reveals another interesting observation. A comparison of the uninoculated controls for auto-oxidation of ascorbic acid with the cultures showing sparing of the vitamin by the various carbohydrates indicates a higher concentration of the vitamin remaining in the cultures than in the controls. It would appear that the increased protection in the presence of enormous numbers of bacteria was due to rapid removal of residual traces of oxygen in the medium by the organisms.

RELATION BETWEEN BACTERIAL GROWTH AND DECOMPOSITION OF VITAMIN C

Vitamin C as a carbon food source in synthetic medium

A series of experiments was designed to inquire into the ability of representative species of vitamin-C-decomposing bacteria to utilize ascorbic acid as sole carbon source in synthetic medium. A basal synthetic broth medium was prepared having the following composition:

NH_4Cl	5.0 gm.
$(NH_4)_2HPO_4$	0.5 gm.
Na_2SO_4	5.0 gm.
$MgSO_4$	0.1 gm.
KH_2PO_4	1.0 gm.
K_2HPO_4	1.0 gm.
Distilled water	1,000.0 ml.

Ascorbic acid was added to the basal medium in concentrations of 10.00, 2.00, 0.8, 0.25, and 0 (control) mg. per ml. For comparative purposes growth was followed in glucose-basal-medium broth and peptone-basal-medium broth having the same concentrations of ascorbic acid as the synthetic medium. All media were buffered to pH 7.0, after adding ascorbic acid.

Four of the test organisms, *Escherichia coli*, *Aerobacter aerogenes*, *Proteus morgani* and *Salmonella aertrycke*, were able to grow in ascorbic-acid-basal broth in concentrations of 0.25, 0.8 and 2.0 mg. per ml., upon first transfer from nutrient broth. Growth was slow in synthetic ascorbic acid medium and, depending on the concentration of the vitamin, required anywhere from 3 to 7 days to develop sufficiently to destroy the vitamin completely. A vitamin concentration of 2 mg. per ml. was definitely inhibitory to all species studied, in spite of buffering of the medium at pH 7.0. Upon repeated transfer in ascorbic-acid-basal broth *Aerobacter aerogenes* was the only species which showed persistent ability to grow and decompose the substance. *Escherichia coli*, *Proteus morgani* and *Salmonella*

aertrycke failed to develop after the second transfer to ascorbic-acid-basal broth, indicating that growth and subsequent decomposition of the vitamin upon initial inoculation to synthetic broth probably was due to sufficient nutrient material that was carried over in the inoculum from the parent nutrient broth culture. The same four organisms were able to grow on continuous transfer in glucose-basal medium in which glucose was the only source of carbon; they also grew well when ascorbic acid was added to the glucose-basal medium in concentrations of 0.25, 0.8 and 2.0 mg. per ml., but growth was inhibited entirely when the vitamin concentration was increased to 10.00 mg. per ml. Glucose in the synthetic broth protected the vitamin completely from oxidation by the test organisms that were able to develop in the medium. *Streptococcus liquefaciens* apparently was unable to grow in the synthetic medium having NH_4Cl as the only nitrogen source, even when glucose was used as a carbon source; likewise, it failed to grow when ascorbic acid was used as the carbon source.

In spite of the buffering of the medium at pH 7.0, a concentration of 10 mg. ascorbic acid per ml. in basal broth or in glucose-basal broth inhibited growth completely in some instances, and actually killed off the inoculum in others; however, the addition of peptone to the media in 1 per cent amount appeared to remove the inhibitory and germicidal effects entirely and, as will be noted later, stimulated tremendous increase in growth over and above that observed in parallel controls which lacked the vitamin.

Influence of concentration of ascorbic acid in peptone broth on bacterial growth

The data in table 3 show a direct correlation between degree of utilization of ascorbic acid by bacteria and degree of growth. As indicated by galvanometer deflection values representing growth, increase in ascorbic acid concentration to the extent of 10.00 mg. per ml. broth brought about a tremendous increase in growth, compared with the vitamin-free controls. All five ascorbic-acid-fermenting species tested showed similar marked stimulation. Since concentration of ascorbic acid directly influences the degree of growth, and since the rate of growth correlates well with the rate of vitamin decomposition, being greatly accelerated when the cells are most viable, utilization of the vitamin as a carbon food source appears quite certain.

Three species of bacteria which are unable to utilize ascorbic acid, namely *Proteus* X-19, *Salmonella pullorum* and *Serratia marcescens*, were not affected significantly by differences in concentration of the substance in peptone broth.

MODE OF DECOMPOSITION

The course of ascorbic acid oxidation varies with the nature of the oxidizing agent. Normal oxidation of vitamin C in plant tissues, catalyzed by enzymes, has been shown by Szent-Gyorgyi (1931) to be a reversible oxidation to the dehydro state. On the other hand, according to Barron *et al.* (1936) and Kellie and Zilva (1935), atmospheric oxidation of the vitamin in an alkaline solution proceeds under the influence of cupric ions to a more complete irreversible stage. Decomposition of ascorbic acid by bacteria also is carried beyond the reversible

dehydro state. As shown in table 1, numerous cultures of various organisms which had removed the entire amount of ascorbic acid added to the medium in the reduced state failed to show any recovery of vitamin C in the reduced form upon treatment with hydrogen sulfide and subsequent removal of the sulfuretted gas with nitrogen. If the oxidation had been merely a reversible one, through the dehydro state, hydrogen sulfide would have reduced the oxidized product, restoring it to its original form.

TABLE 3

Influence of concentration of ascorbic acid in peptone broth buffered at pH 7.0 upon growth of ascorbic acid-decomposing and on ascorbic acid-non-decomposing bacteria

ASCORBIC ACID-DECOMPOSING SPECIES INCUBATED UNDER ANAEROBIC CONDITIONS AT 37°C.	CONCENTRATION OF ASCORBIC ACID								
	0.0 mg./ml.			0.8 mg./ml.			10.00 mg./ml.		
	0 hours	10 hours	36 hours	0 hours	10 hours	36 hours	0 hours	10 hours	36 hours
Escherichia coli (Dd)	5*	30	35	5	44	57	5	50	58
				+†	−	−	+	+	−
Aerobacter aerogenes (5A5)	6	20	37	5	33	44	5	47	82
				+	−	−	+	+	−
Proteus morgani (Ba)	3	15	40	4	31	50	4	33	74
				+	−	−	+	+	−
Salmonella aertrycke	2	19	23	2	34	44	2	57	76
				+	−	−	+	+	−
Streptococcus liquefaciens	3	19	19	2	34	34	4	49	78
				+	−	−	+	+	−
Non-decomposing species									
Proteus X-19	3	11	12	4	7	10	4	7	10
				+	+	+	+	+	+
Salmonella pullorum (P-54)	4	15	16	3	12	16	7	10	10
				+	+	+	+	+	+
Serratia marcescens (C-20)	2	13	14	4	12	14	4	12	14
				+	+	+	+	+	+

* Units galvanometer deflection of Evelyn photoelectric colorimeter indicating turbidity and indirectly degree of growth.

† + indicates presence of ascorbic acid in medium. − indicates complete bacterial decomposition of the vitamin.

Gas production during the fermentation of carbohydrates usually indicates a rather complete decomposition process. Accordingly, all of the species showing ability to decompose ascorbic acid were inoculated into peptone ascorbic-acid broth contained in large Durham fermentation tubes, to determine the production of gas during the decomposition process. Concentrations of 4.0 and 2.0 mg. ascorbic acid per ml. broth were employed, and all cultures incubated in duplicate under anaerobic conditions at 37°C. As shown in table 1, organisms which

usually produce gas in carbohydrate medium (members of the colon-aerogenes and *Salmonella* groups) also generate gas during the fermentation of ascorbic acid; on the other hand, species of *Streptococcus* and *Vibrio*, as well as *Eberthella typhosa* and *Salmonella gallinarum*, which are normally non-aerogenic, likewise failed to produce gas during the oxidation.

Periodic quantitative tests for ascorbic acid in the duplicate culture series showed a positive correlation between increase in gas production and decrease in the vitamin content of the medium. The amount of gas production also directly corresponded with the amount of vitamin substrate added to the medium. Parallel control cultures lacking the vitamin substrate showed no gas production.

Dehydrogenase studies. Filtrates from heavy cultures of vitamin-C-decomposing bacteria showed no effect on ascorbic acid under either aerobic or anaerobic conditions, regardless of age of culture; thus it seems that the factor responsible is not an exoenzyme. The present studies have demonstrated that the decomposition occurs rapidly under anaerobic conditions in the presence of living, actively metabolizing cells, and that the activation of the substrate in the absence of molecular oxygen implies the operation of a dehydrogenase system.

The "initial capacity" of the organisms to attack ascorbic acid and the "all or none" type of the response suggest that the endoenzyme involved is constitutive rather than adaptive in character. A series of experiments was carried out in which both vitamin-C-decomposing and non-decomposing species of bacteria were exposed to ascorbic acid substrate in nutrient broth by frequent transfer for varying periods up to three and one-half months. It was found that daily transfer of vitamin-C-decomposing species over a period of only five days resulted in marked acceleration of the *rate* of decomposition, but this "adaptive" characteristic was only temporary. Non-decomposing species exposed to ascorbic acid substrate over a period of several weeks failed to respond adaptively.

The operation of a dehydrogenase system may be detected easily under anaerobic conditions by the use of an appropriate oxidation-reduction dye indicator, the substrate in question and washed "resting cells" of the bacterial species to be examined. Accordingly, an experiment was planned to detect transfer of hydrogen by bacteria from ascorbic acid substrate to a dye-indicator acting as hydrogen acceptor. The study of catalytic transfer of hydrogen by bacteria usually is carried out by the use of the well-known technique of Thunberg in which methylene blue is employed as artificial hydrogen acceptor. In selecting an oxidation-reduction indicator for the present study it was found that methylene blue could not be used, because it is reduced by the reduced form of ascorbic acid, the rate of reduction depending on the concentration of the reagents involved and the temperature at which the reaction takes place. It was observed also that methylene blue in the concentration required was toxic to some of the test organisms, especially the enterococci. Nile blue proved to be a satisfactory dye-indicator, since it is stable toward large concentrations of ascorbic acid, and in relatively low dilution is not toxic to bacteria. The value of oxidation-reduction dye-indicators like methylene blue and nile blue lies in the fact that these dyes exist in a reduced form which is colorless and in an oxidized form which is blue. Optimum concentrations of the reagents employed were determined by

preliminary experiments, and tests with appropriate controls were carried out under anaerobic conditions at 37°C. A positive test was indicated by complete reduction of nile blue to the colorless state within 60 minutes, providing the parallel control lacking vitamin substrate was not also reduced in that period of time.

The results of the nile blue reduction studies, presented in table 1, show that all of the species of bacteria which are able to decompose ascorbic acid in nutrient broth also activate the vitamin under "resting cell" conditions in the presence of nile blue, thus suggesting the action of an ascorbic-acid dehydrogenase. However, the following organisms which did not attack ascorbic acid in nutrient broth activated the substrate in the presence of nile blue acceptor and reduced the dye: various strains of *Salmonella pullorum*, *Proteus vulgaris*, *Serratia marcescens*, and species of *Bacillus*. Parallel tests in which glucose was used as substrate gave similar results with all organisms tested; however, the rate of reduction was usually a little more rapid than when ascorbic acid was employed as substrate. Most reductions occurred within 20–30 minutes.

From the present observations one might postulate that nile blue acts as a readily available hydrogen acceptor in the oxidation of vitamin C by organisms such as *Salmonella pullorum* and *Proteus vulgaris*, which are unable to attack the substance under ordinary conditions in nutrient medium. Perhaps these organisms do not provide the appropriate hydrogen acceptor or other essential component in the dehydrogenase system, under the conditions of growth in a nutrient medium. Failure of members of the *Alcaligenes* group to activate either glucose or ascorbic acid substrate is in keeping with their asaccharolytic nature and apparent deficiency in dehydrogenase enzyme systems.

Experiments were carried out to determine the effect of various enzyme inhibitors and growth-inhibiting substances on the activation of ascorbic acid in the resting-cell nile-blue system. Sodium fluoride, arsenious oxide, urethane and sodium selenite have been reported by different investigators as arresting the action of various types of dehydrogenases, especially sodium selenite, which seems to affect specifically the dehydrogenases. Sodium selenite (0.01M; 0.001M; 0.0001M) inhibited completely the action of ascorbic acid dehydrogenase of three test organisms, *Escherichia coli*, *Aerobacter aerogenes*, and *Proteus morgani*; viability tests showed that sodium selenite in the concentrations employed does not prevent the growth of these organisms. Potassium cyanide (0.01M; 0.001M), sodium fluoride (0.01M; 0.001M), arsenious oxide (0.02M; 0.002M) and urethane (0.03M; 0.02M) failed to inhibit dye-reduction significantly. These substances when added to nutrient broth in similar concentration did not prevent bacterial growth, but in some instances growth was retarded, depending on the concentration of the inhibitor. Vitamin C added to nutrient broth containing these substances in similar amount was completely destroyed in every case, after good growth was evident, indicating again no inhibition of ascorbic acid activation in nutrient broth. Acetone (5 per cent; 2.5 per cent), toluene (5 per cent; 2.5 per cent) and chloroform (5 per cent; 2.5 per cent) inhibited the reduction of nile blue by the three test species in the presence of ascorbic acid; viability tests showed these agents to be inhibitory to growth or germicidal in the concentrations indicated.

In the light of the present observations, it appears that sodium selenite specifically inhibits ascorbic acid dehydrogenase; even though the substance was not toxic to the cells, it prevented the action of the enzyme. The cells must be living, but not necessarily multiplying, to effect activation of ascorbic acid dehydrogenase in the nile blue system; killing the cells with chemical agents prevented activation, whereas holding the cells at 46°C. to prevent cell growth greatly increased the rate of reduction. On the other hand, under the conditions common to bacterial decomposition of vitamin C in nutrient broth, the cells must be living and also actively multiplying. The following findings support this assumption: (1) holding heavy cell suspensions at 46°C. for several days, to prevent growth without extensive killing of the cells, showed no decomposition of the substance; (2) freezing and thawing massed cells several times, bringing about death and disruption of the majority of the cells, failed to accelerate the oxidation when the cells were resuspended in ascorbic acid nutrient broth; (3) when incubated at 37°C. an incubation period of at least 10–15 hours was required for complete destruction, regardless of the amount of inoculum added; and (4) any chemical factor which prevented or retarded growth of the organisms also prevented or retarded the oxidation of vitamin C.

DISCUSSION AND SUMMARY

The property of decomposing vitamin C is quite common among the enteric bacteria, including the intestinal streptococci.

In the presence of appreciable amounts of easily fermented carbohydrate, like glucose, the vitamin is protected from microbic decomposition, whereas in the absence of the competitive agents the ascorbic acid content of the medium becomes depleted rapidly.

Bacterial decomposition proceeds under anaerobic conditions. Bacteria which do not attack the vitamin exert a sparing action on this substance under ordinary aerobic conditions by removing atmospheric oxygen, and thus preventing auto-oxidation. Usually, such organisms grew better in the presence of the vitamin than in its absence, due presumably, at least in part, to the creation by this chemical agent of a lower and more favorable reduction potential.

Ascorbic acid was decomposed and utilized readily as a carbon food for the attacking bacteria, when the medium contained a suitable source of organic nitrogen, like peptone. This was indicated by the early disappearance of the vitamin and the large increase in bacterial growth over that observed in parallel vitamin-free controls. In the absence of a suitable nitrogen source, as for example in synthetic ammonium-chloride ascorbic-acid medium containing 10 mg. per ml. of the agent, no growth took place, and the bacterial cells were killed, in spite of buffering of the medium at pH 7.0. At lower concentrations of ascorbic acid (2 mg. or less per ml.) *Aerobacter aerogenes* alone was able to develop and decompose the substance.

Oxidation of the vitamin was in every instance carried beyond the reversible dehydro stage of decomposition. Furthermore, vitamin-C-decomposing bacteria which produced gas from ordinary carbohydrates also brought about gas formation in their action upon ascorbic acid.

Filtrates of active bacterial cultures did not possess the vitamin-C-decomposing property, the destruction being effected only by actively metabolizing microbial cells. These observations, together with the demonstration that the carrying to completion of the process in the absence of molecular oxygen, pointed to the operation of a dehydrogenase in the destruction of the vitamin. A dehydrogenase was demonstrated by the use of bacterial "resting cells" in a nile-blue indicator system having ascorbic acid as the substrate.

Results of the present study lend further support to the assumption that under certain conditions vitamin C may be destroyed in the intestinal canal by bacteria, and thus lead to vitamin C deficiency. It would seem that this action cannot be of serious moment in the population at large, otherwise there would be more extensive clinical evidence of such deficiency. No doubt such factors as the small daily amount of ascorbic acid required by the normal human adult, the site and the rapidity of absorption from the intestine, enteric infection, diet, type of intestinal flora and dominance of vitamin-C-destroying bacteria, are definitely related to loss to the body by bacterial action. It may well be that bacterial destruction of ascorbic acid in the intestine becomes especially significant in certain pathological conditions where the vitamin, in addition to its essential rôle in normal metabolism, serves to play an effective part in recovery. Thus, in the healing of bone fractures or wounds in general, in certain conditions of shock, hypersensitivity, infection and poisoning due to various industrial chemicals the bodily demand for vitamin C is so increased that bacterial destruction of the substance and loss to the body by perspiration or by any other factor becomes exceedingly important (see Holmes, 1942).

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