The fact that the interatomic distances fall below the sum of the tetrahedral radii has been interpreted as due to multiple bond formation and to ionic character. These two effects may both lead to decreases in interatomic distances in XO_4^{m-} ions but they lead to opposite effects on the negativity of the oxygen atoms. Thus a measure of the relative importance of the two factors might be obtained by measurement of the length of hydrogen bonds.

In the present case if it is assumed that the O–H bond is the same in the arsenate and phosphate groups, then the negativity of the oxygen atoms would also appear to be very nearly the same. A more definite statement can scarcely be made until the possible influence of randomness on the parameters of KH_2AsO_4 is investigated.

Summary

The atomic parameters for the crystals potassium dihydrogen arsenate and silver arsenate have been determined. The arsenic to oxygen distance in both crystals has been found to be 1.75 Å. within the limits of error.

The silver to oxygen distance (2.34 Å.) in silver arsenate is discussed in connection with the colors of this compound and others containing silver to oxygen bonds and in the light of the suggestions of Pitzer and Hildebrand concerning color and covalent character.

The influence of randomness on the determination parameters of potassium dihydrogen phosphate is discussed. The hydrogen bond distance (O–H–O) in this crystal is found to be 2.54 Å., equal to that in the phosphate.

HANOVER, N. H. RECEIVED NOVEMBER 7, 1941

[CONTRIBUTION FROM DEPARTMENT OF DAIRY INDUSTRY AND BACTERIOLOGY, NEW YORK STATE COLLEGE OF AGRICULTURE, CORNELL UNIVERSITY]

Oxidation and Reduction of Vitamin C[1]

BY DAVID B. HAND AND ELIZABETH CHASE GREISEN

Introduction

The oxidation of vitamin C by means of various catalysts was investigated primarily to see whether the reaction could be used in the determination of oxygen in biological materials. It was further hoped that additional information on oxidation and reduction of vitamin C would in a general way contribute to our knowledge of the role of vitamin C in biological oxidations and its stability in food products.

Effect of Catalysts on the Amount of Oxygen Used in the Oxidation of Vitamin C.—The amount of oxygen used in the oxidation of vitamin C depends on the nature of the catalyst. With vitamin C oxidase from cucumbers exactly one atom of oxygen is used per molecule of vitamin C. This result has been approximately confirmed by the earlier work of Snow and Zilva[2] and Ebihara.[3] The ratio has been determined accurately by us in order to make possible a calculation of dissolved oxygen from measured disappearance of reduced ascorbic acid. The average of twelve determinations made with a differential manometer (Barcroft) was 0.995 atom of oxygen per molecule of vitamin C with a mean deviation of ±1.34% and a maximum deviation of ±3.0%. This ratio was determined in milk at a *p*H of 6.6. Experiments made in buffer solutions from *p*H 4.96 to 7.11 indicated that the ratio is independent of *p*H in this range, provided the amount of enzyme is large enough so that catalysis by metallic impurities in the buffers is negligible.

When the oxidation of vitamin C is catalyzed photochemically in the presence of riboflavin, almost two atoms of oxygen are used by each molecule of vitamin C. Table I shows a summary of the results. The fact that variable amounts of oxygen are used in the reaction makes the photochemical oxidation of vitamin C unsuitable for determining dissolved oxygen. When copper acts as catalyst about 1.5 atoms of oxygen combine with one molecule of vitamin C.

The explanation for the combination with more than one atom of oxygen lies in the intermediate formation of hydrogen peroxide and its further reactions. If the hydrogen peroxide oxidizes ascorbic acid or is decomposed by catalase the number of oxygen atoms equals one; but if

(1) Paper presented before Cincinnati meeting of American Chemical Society, April, 1940.

(2) G. A. Snow and S. S. Zilva, *Biochem. J.*, **32**, 1926–1937 (1938).

(3) T. Ebihara, *Japanese J. Biochem.*, **29**, 199 (1939).

TABLE I

OXYGEN CONSUMPTION BY VITAMIN C WITH COPPER, RIBOFLAVIN, AND ALKALI AS CATALYSTS (ATOMS OF OXYGEN PER MOLECULE OF VITAMIN C)

Buffer		Cucumber oxidase	Copper	Riboflavin and light	Alkali
Phthalate *p*H	3.91	...	1.45	2.04	..
Phthalate *p*H	4.93	0.988	1.52	2.07	..
Phthalate *p*H	5.56	0.979	1.40	1.57	..
Phosphate *p*H	6.89	...	1.19	1.85	..
Phosphate *p*H	7.11	1.00	..	..	..
Phosphate *p*H	7.72	...	1.43	1.88	..
Borate *p*H	10.15	...	1.67	..	2.00
Milk	6.6	0.995	1.34	..	..

EXPERIMENTAL CONDITIONS FOR FIG. 1 AND TABLE I

Differential Manometer (Barcroft).—Calibration as described by Dixon,[4] 26°, shaking rate 120 per minute, fluid volume 5 cc., gas volume approximately 30 cc. Catalysts put in side arm and added after temperature equilibration and zero readings taken. *p*H determinations with glass electrode.

Ascorbic Acid (Pfizer).—100.01% pure, average of five titrations with sodium hydroxide, maximum deviation +0.69%.

Oxidation by Riboflavin.—0.05 mg. of riboflavin, 2.0 cc. of 0.010 *M* buffer, 4.0 mg. of ascorbic acid, condensed light from capillary mercury arc.

Oxidation by Riboflavin in Presence of Catalase.—0.1 mg. of riboflavin, 0.1 cc. of catalase concentrate from horse liver, 1.0 cc. of 0.05 *M* phthalate buffer, *p*H 5.28; 2.0 mg. of ascorbic acid; scale of time reduced to one-half in Fig. 1.

Oxidation by Copper.—0.2 mg. of $CuSO_4 \cdot 5H_2O$ (1.0 mg. needed to give corresponding velocity of reaction in milk); 2.0 cc. of 0.10 *M* buffer; 4.0 mg. of ascorbic acid.

Oxidation by Cucumber Oxidase.—(a) In milk: winter storage cucumbers were frozen and thawed before squeezing to obtain juice and 1.0 cc. used; whole milk pretreated by warming to 42° and boiling under vacuum for one minute to remove carbon dioxide, 3 cc. used; *p*H 6.6; 4.0 mg. of ascorbic acid. The correction for ascorbic acid in 3 cc. of milk was 0.0495 mg. Control experiments with cucumber juice and vitamin C-free milk showed no oxygen uptake. (b) In buffer: 1.0 cc. of cucumber juice; 2.0 cc. of 0.10 *M* buffer; 4.0 mg. of ascorbic acid.

the hydrogen peroxide accumulates in the solution or oxidizes dehydroascorbic acid the number of oxygen atoms equals two.

The experimental evidence for the above conclusions was the following. Hydrogen peroxide was found in the solutions after the oxidation of ascorbic acid was complete. The tests were made by iodometric titration, and also by adding catalase and measuring the volume of oxygen liberated. Although easily measured, the amounts of hydrogen peroxide were only a fraction of the theoretical. When the oxidation was carried out in the presence of catalase the oxygen combining power of vitamin C was decreased as shown in Fig. 1.

(4) M. Dixon, "Manometric Methods," Cambridge Press, 1934, pp. 30, 37.

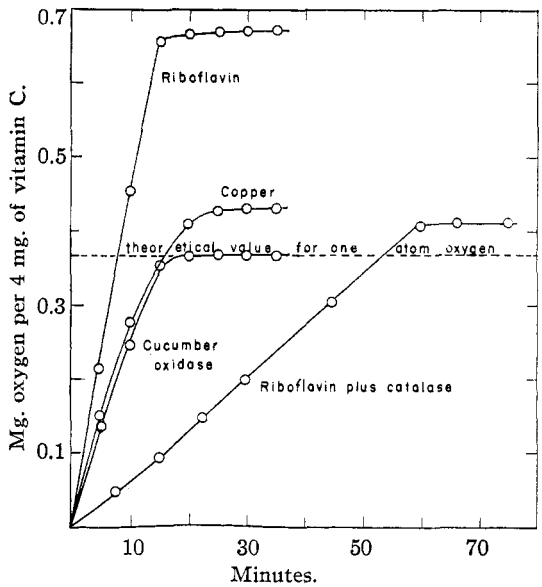


Fig. 1.—Effect of catalyst on the reaction between vitamin C and oxygen.

Under conditions in which hydrogen peroxide was produced the oxidation of ascorbic acid was partly irreversible. The oxidation by copper was more reversible and less hydrogen peroxide could be found than in the case of the photochemical oxidation. With the vitamin C oxidase the oxidation was completely reversible and no hydrogen peroxide could be observed (see Table II).

TABLE II

EFFECT OF CATALYST ON THE REVERSIBILITY OF VITAMIN C OXIDATION

Experimental conditions: oxidation carried out at *p*H 6.0 in 0.05 *M* phthalate buffer, 20°, 200 mg. of ascorbic acid per liter; fifty cc. of cucumber juice, 1 mg. of $CuSO_4 \cdot 5H_2O$, 2 mg. of riboflavin per liter; sunlight. Reduction with bacteria carried out at *p*H 6.5 by adding 5 cc. of dehydroascorbic acid solution to 5 cc. of skim milk containing 0.5 cc. of washed *B. coli* suspension and holding thirty minutes at 40°. Reduction with hydrogen carried out by adding 2 mg. of riboflavin and 10 cc. of colloidal platinum black per liter and bubbling hydrogen through solution for ten hours at 20°.

	Per cent. reversibility	
	B. coli	Hydrogen
Cucumber oxidase	99	58
Copper	90	20
Light and riboflavin	83	13

The difference between copper and light in their effects on the oxygen-combining power of vitamin C can be accounted for by the reasonable assumption that copper catalyzes the oxidation of ascorbic acid by hydrogen peroxide. Our results with

copper are approximately but not exactly in agreement with those of Barron, Klemperer, and DeMeio,[5] who reported that below *p*H 6.6 one atom of oxygen reacted with one molecule of vitamin C with copper as catalyst. In discussing the mechanism of the reaction these authors postulated the intermediate formation of hydrogen peroxide and its instantaneous decomposition by the copper ion.

At *p*H 10.15 in borate buffer 2 atoms of oxygen are used in the oxidation of 1 molecule of vitamin C. If copper ion is present the oxygen consumption falls to 1.67. It is stated in the literature[6] that as many as 3 atoms of oxygen are used in alkaline solutions. Utilization of more than 2 atoms of oxygen should involve oxidation of threonic acid.[7]

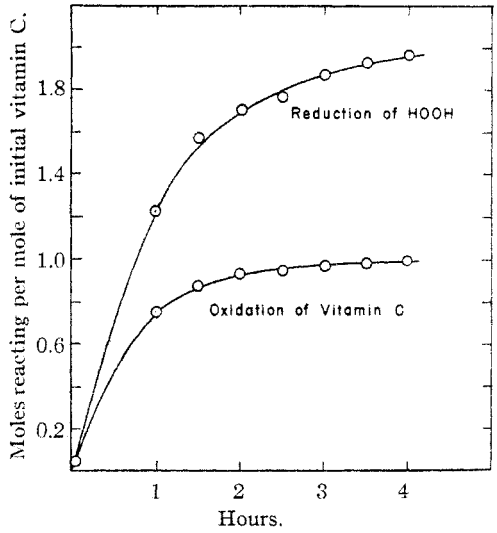


Fig. 2.—Reaction between vitamin C and hydrogen peroxide.

Interaction of Vitamin C and Hydrogen Peroxide.—The interaction of vitamin C and hydrogen peroxide was studied separately. A method of analysis was worked out by which vitamin C and hydrogen peroxide could be determined in the presence of each other without interference. The vitamin C was first titrated with indophenol dye by the customary procedure in dilute sulfuric acid. Then a few crystals of potassium iodide were added to the acid solution, and the iodine liberated by hydrogen peroxide was titrated with 0.01 *N* thiosulfate after standing for fifteen minutes.

In a typical experiment, vitamin C and hydrogen peroxide were mixed to give 200 cc. of a solution which was 0.00453 normal with vitamin C, 0.0169 normal with hydrogen peroxide and 0.05 molar with phthalate buffer of *p*H 6.0. All dissolved oxygen was drawn out of the flask, which was kept under vacuum at 25°. Separate controls were run with hydrogen peroxide and vitamin C alone. In these decomposition and oxidation were negligible. Figure 2 shows the reaction between vitamin C and hydrogen peroxide when catalyzed presumably by the metallic impurities in the phthalate buffer. The results are plotted in moles per mole of vitamin C. The oxidation of the vitamin C was practically complete in four hours, the initial concentration of 0.00453 falling to 0.00014 molar. During the same time the hydrogen peroxide fell from 0.0169 to 0.00827 molar. For each mole of vitamin C about 2 moles of hydrogen peroxide were used.

As seen in the figure the second step in the oxidation of vitamin C by hydrogen peroxide is almost instantaneous. Since the second stage in the oxidation by hydrogen peroxide is undoubtedly irreversible, one would expect only partial reversibility in the oxidations catalyzed by riboflavin and copper ion.

Reduction of Dehydroascorbic Acid.—We have found that dehydroascorbic acid can be reduced by hydrogen if both platinum black and riboflavin are present in the solution. The riboflavin is reduced very rapidly and it then reacts slowly with the dehydroascorbic acid. This is an interesting interaction between riboflavin and vitamin C which calls to mind the peculiar role of riboflavin in the photochemical oxidation of vitamin C.[8] Several authors have reported unsuccessful attempts to reduce dehydroascorbic acid with hydrogen and platinum catalyst.[5,9,10]

We have attempted to use the reaction as a basis for chemical determination of total vitamin C (ascorbic acid plus dehydroascorbic acid). These experiments were unsuccessful because of the gradual inactivation of the platinum catalyst

(5) E. S. G. Barron, R. H. DeMeio and F. Klemperer, *J. Biol. Chem.*, **112**, 625–640 (1935).

(6) R. D. H. Heard and A. and M. Welch, *Biochem. J.*, **29**, 998 (1935).

(7) H. Borsook, H. W. Davenport, C. E. P. Jeffreys and R. C. Warner, *J. Biol. Chem.*, **117**, 237 (1936).

(8) D. B. Hand, E. S. Guthrie and P. F. Sharp, *Science*, **87**, 439 (1938).

(9) C. G. Daubney and S. S. Zilva, *Biochem. J.*, **20**, 519 (1926).

(10) J. Tillmans, P. Hirsch and H. Dick, *Z. Untersuch. Lebensmittel*, **63**, 267 (1932).

during the reduction. In some cases reduction was complete in from two to ten hours but frequently reduction stopped before it was complete.

A convenient method for reducing dehydroascorbic acid is with bacteria of the *B. coli* group.[11] Using a few drops of cell suspension per 10 cc. of dehydroascorbic acid solution, reduction is complete in fifteen minutes at 40°. The details of the procedure have been described in another publication.[12]

Experiments made on the reversibility of the oxidation of vitamin C using *B. coli* and glucose as the reducing agent showed that reversibility also varied with the nature of the catalyst The results are shown in Table II.

(11) W. B. Esselen, Jr., and J. E. Fuller, *J. Bact.*, **37**, 501 (1939).
(12) I. C. Gunsalus and D. B. Hand, *J. Biol. Chem.*, **141**, 853 1941).

Summary

The amount of oxygen used in the oxidation of vitamin C depends on the nature of the catalyst: with cucumber oxidase 1.0, with copper 1.19 to 1.67, and with riboflavin in the light 1.57 to 2 atoms of oxygen are involved. The oxidation is completely reversible with 1.0 but only partially reversible with more than 1.0 atom of oxygen. The partial irreversibility is due to the production of hydrogen peroxide. Two molecules of hydrogen peroxide oxidize one molecule of ascorbic acid.

ITHACA, N. Y. RECEIVED SEPTEMBER 22, 1941

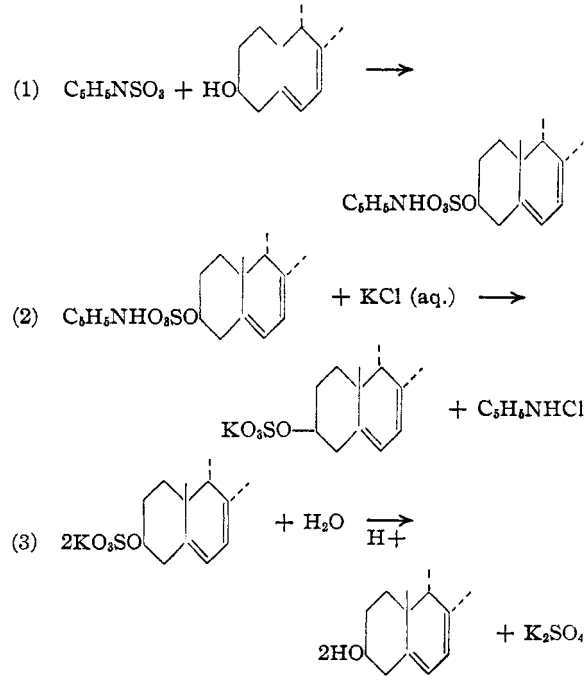
[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE POLYTECHNIC INSTITUTE OF BROOKLYN, BROOKLYN, NEW YORK, AND THE PEDIATRIC RESEARCH LABORATORY OF THE JEWISH HOSPITAL OF BROOKLYN]

Steryl Sulfates. II. Isolation and Separation of Sterols

BY ALBERT E. SOBEL[1] AND PAUL E. SPOERRI

As pointed out in the first paper of this series[2] the preparation of steryl sulfates may be of aid in the isolation and separation of sterols. The properties that recommend the sulfate derivatives for this purpose are: (1) ease of formation in quantitative yields, (2) insolubility in lipid solvents, (3) inexpensiveness when compared to digitonides, which are commonly employed because of their insolubility in lipid solvents.

An illustration of the usefulness of the sulfate derivative for the separation of sterols is the complete removal of cholesterol from the reaction products of the thermal decomposition of calcium and potassium cholesteryl sulfates. Previously, the costly digitonin would have been employed in such a process. A second application of the sulfate derivative is the quantitative removal of cholesterol from cholesteryl acetate. Here, again, digitonin would have been employed in the past. A third application is the successful isolation of ergosterol from a natural product, *i. e.*, brewer's yeast. The lipid fraction of the yeast was isolated by refluxing with hot alcohol in order to break up the combined form of ergosterol (probably in combination with protein[3]) and by extracting with ether. The combined extracts were then evaporated to dryness. Attempts made to use this product directly were unsuccessful. Therefore, the oil was purified by extracting it with petroleum ether and discarding the insoluble portion. The lipid extracts were then saponified and the ergosterol isolated by the following reactions



(1) From the dissertation submitted to the Polytechnic Institute of Brooklyn in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June, 1940.
(2) A. E. Sobel and P. E. Spoerri, THIS JOURNAL, **63**, 1259 (1941).
(3) C. E. Bills, *Physiol. Rev.*, **15**, 1 (1935).