all evidence indicates that isolated nuclei are very permeable to P_i.

It must be noted that Rees and Rowland (1961) and Rees *et al.* (1962) previously postulated that liver and kidney nuclei possess a phosphorylative system. The significance of their data was obscured in part however because they used preparations of nuclei containing demonstrable levels of mitochondrial contamination. It is impossible to draw any concrete conclusions at this time concerning the nature of the phosphorylation catalyzed by liver nuclei, but our results verify that they do possess an energy-transduction system. The results provide substance to the postulations by the English investigators. Work is in progress on the nature of the phosphorylation products formed in the presence of the various nucleosides.

References

Allfrey, V. G. (1963), *Proc. Intern. Congr. Biochem. 5th, Moscow, 1961, 2,* 127.

Caputto, R. (1962), *Enzymes 6,* 133.

Creasey, W. A., and Stocken, L. A. (1959), *Biochem. J. 72,* 519.

Löw, H. (1959), *Biochim. Biophys. Acta 32,* 1, 11.

McEwen, B. S., Allfrey, V. G., and Mirsky, A. E. (1963), *J. Biol. Chem. 238,* 758, 2571, 2579.

Osawa, S., Allfrey, V. G., and Mirsky, A. E. (1957), *J. Gen. Physiol. 40,* 491.

Penniall, R., Hall, A. H., Griffin, J. B., and Irvin, J. L. (1962), *Arch. Biochem. Biophys. 98,* 128.

Penniall, R., Liu, S. M., and Saunders, J. P. (1963), *Biochim. Biophys. Acta 76,* 170.

Penniall, R., Saunders, J. P., and Liu, S. M. (1964), *Biochemistry 3,* 1454 (this issue; preceding paper, paper II of this series).

Rees, K. R., and Rowland, G. F. (1961), *Biochem. J. 78,* 89.

Rees, K. R., Ross, H. F., and Rowland, G. F. (1962), *Biochem. J. 83,* 523.

Siebert, G. (1963), *Proc. Intern. Congr. Biochem. 5th, Moscow, 1961, 2,* 93.

Weinbach, E. C. (1959), *J. Biol. Chem. 234,* 1580.

A Monodehydro Form of Ascorbic Acid in the Autoxidation of Ascorbic Acid to Dehydroascorbic Acid

NICHOLAS G. LEVANDOSKI,* EUGENE M. BAKER, AND JOHN E. CANHAM

From the U. S. Army Medical Research and Nutrition Laboratory, Fitzsimons General Hospital, Denver, Colorado

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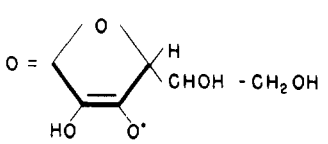
The existence of monodehydroascorbic acid in the oxidative pathway of L-ascorbic acid to dehydroascorbic acid was suggested by available literature. Experiments performed in this laboratory have now led to the isolation and characterization of this intermediate compound. Aqueous solutions of L-ascorbic acid and L-[1-^{14}C]ascorbic acid were chromatographed using descending chromatography. Kinetic data obtained using the ^{14}C-labeled ascorbate places the intermediate in the pathway as well as establishing the rate of intermediate formation. These studies indicate that this intermediate is monodehydroascorbic acid and may be complexed with ascorbic acid.

The existence of ascorbic acid in a monodehydro form as a free radical (I) is assumed in order to support the theoretical role of L-ascorbic acid in intermediary metabolism such as hydroxylation reactions. This



Structural Formula I

postulate necessitates a one-electron-transport system present in the oxidative pathway of L-ascorbic acid to dehydroascorbic acid. Recent work (Finholt *et al.*, 1963) has supported the existence of a monodehydro form of the acid, based on the maximum in the *p*H-rate profile at $pH = pK_a$ of ascorbic acid and on the observed first-order characteristics of the reaction. Other work (Barr and King, 1956) on the γ-ray-induced oxidation of ascorbic acid was interpreted in terms of radical mechanisms. The absence of a chain utilization of oxygen by ascorbic acid is consistent with the known protective effect on biological systems (Géro and Le Gallic, 1952). The compounds that exert this effect are characterized by their ease of oxidation and by their formation of stable one-electron oxidation products. The remainder of the oxidative pathway, the decomposition of dehydroascorbic acid to 2,3-diketogulonic acid and the elucidation of the Browning-reaction products, has recently been reported (Kamiya, 1960a).

Experiments (Baker *et al.*, 1963) dealing with the catabolism of L-[1-^{14}C]ascorbic acid in man have shown that changes in $^{14}CO_2$ expiration occurred with degradative changes of the acid. Work was initiated to identify the intermediates in the pathway responsible for these changes (Levandoski *et al.*, 1963). This paper centers on the identification and characterization of a proposed monodehydroascorbic acid–ascorbic acid complex as one of the intermediates. Work with other organic acids has shown that the acid molecule can combine with its own ion to form a charge-transfer complex. This acid-anion complex is seen in adipic acid, *p*-hydroxybenzoic acid, dihydroxybenzoic acid, and phenylacetic acid (Finholt *et al.*, 1963). Data from this laboratory suggest that a radical transfer complex may exist with monodehydroascorbic acid–ascorbic acid.

Materials and Methods

The three sources of L-ascorbic acid used in this study were: L-[1-^{14}C]ascorbic acid with a specific activity of 1.65 mc/mM obtained from California Corp. for

* Present address: Department of Chemistry, University of Colorado, Boulder, Colorado.

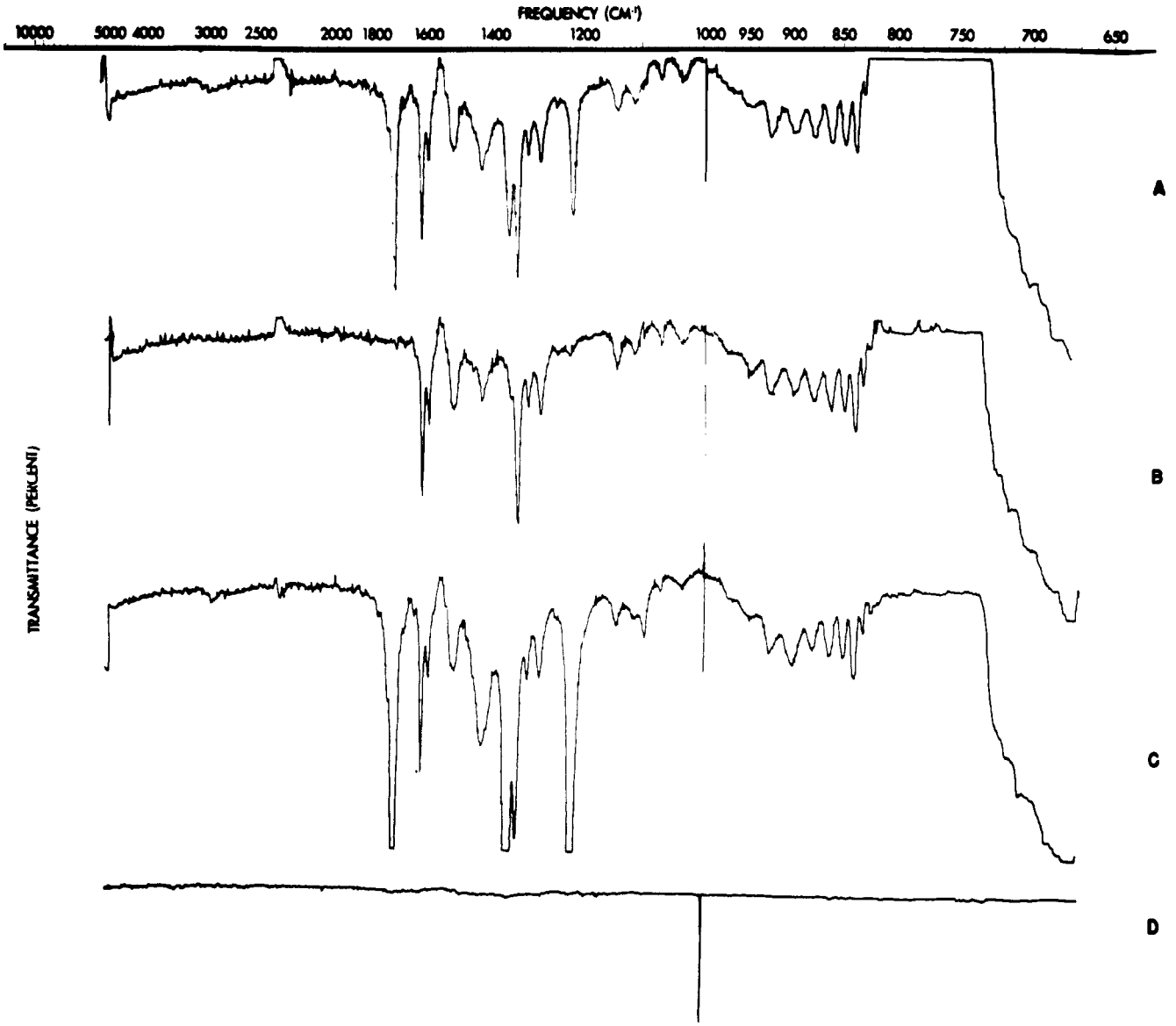


FIG. 1.—Infrared spectrum of DNPH derivatives (CCl_4-0.1%). (A) Derivative of L-ascorbic acid; (B) derivative of 2,3-diketogulonic acid; (C) derivative of MDHAA-AA; (D) baseline scan, double beam CCl_4.

Biochemical Research; USP Ascorbic Acid Reference Standard distributed by the Board of Trustees of the United States Pharmacopeial Convention, Inc.; and L-ascorbic acid powder (lot 25059) obtained from J. T. Baker Chemical Co. The homogeneity and purity of each ascorbic acid sample used was checked, immediately after dissolving in water, by determining the specific rotation and by paper chromatography. The specific activity and purity of the L-[1-^{14}C]ascorbic acid was checked by radioassay and autoradiography. Water used in preparing the solutions used in this study was demineralized triple-glass-distilled water registering less than 0.1 ppm as NaCl (Corning Still). Descending paper chromatography was used with Whatman No. 1 paper as the stationary phase, and the solvent systems of phenol–acetic acid–water (100:1:100) solvent system A (Mapson and Partridge, 1959), and 1-butanol–acetic acid–water (250:60:250) solvent system B (Tegethoff, 1953), in a hydrogen sulfide atmosphere. The spray reagent used was 2,6-dichlorophenolindophenol, prepared by dissolving 800 mg of the dye in 1 liter of water followed by dilution with an equal volume of ethanol.

Melting points were taken on a Fischer-Johns apparatus and corrected. Elemental analysis was obtained on a F & M carbon, hydrogen, nitrogen analyzer, Model 180. The oxidation of the compounds was catalyzed with silver oxide at an oven temperature of >800°.

Intermediates were isolated by preparative chromatography using solvent system B and a 20-hour development. The chromatograms were dried in a nitrogen atmosphere. A parallel marker was sprayed with 2,6-dichlorophenolindophenol, and the appropriate untreated areas of the chromatogram were eluted with 30–40 ml. of triple-distilled demineralized water, shell frozen, and lyophilized.

EXPERIMENTAL

Monodehydroascorbic acid–ascorbic acid (MDHAA-AA)[1] isolated from the band at R_F 0.12 (solvent system B) when lyophilized to dryness was found to be extremely unstable when exposed to air at room temperature but was more stable under nitrogen. Upon fur-

[1] Abbreviations used in this work: MDHAA-AA, monodehydroascorbic acid–ascorbic acid; DNPH, 2,4-dinitrophenylhydrazine.

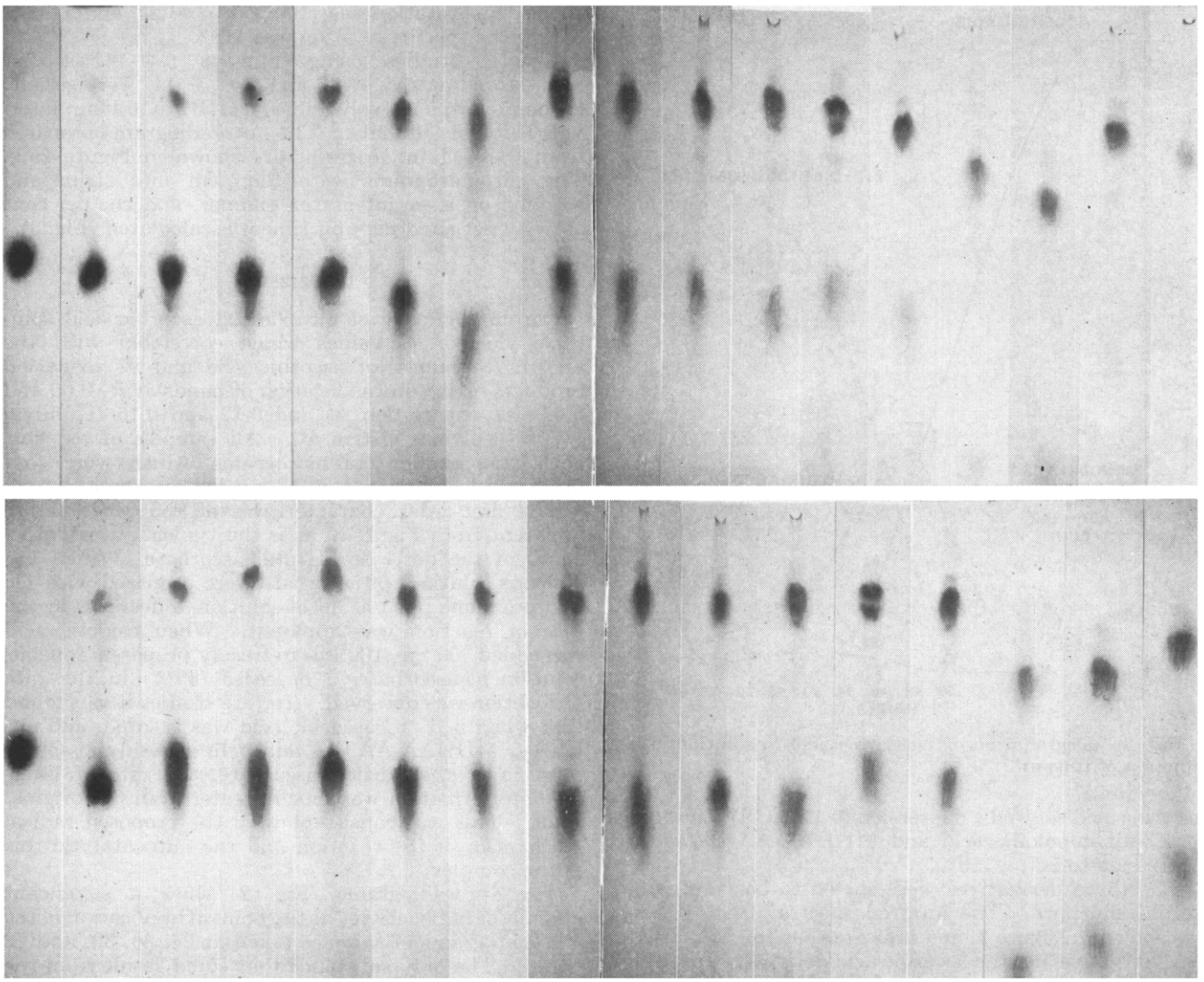


FIG. 2.—Serial autoradiograms of aqueous L-ascorbic acid solution, chromatographed at 6-hour intervals. (a, upper) from left, 0–102 hours, stored at 25° ± 1°; (b, lower) from left, 0–108 hours, stored at 37° ± 1°.

ther decomposition chromatograms of MDHAA-AA showed significant amount of tailing between R_F 0.12 and R_F 0.37 (ascorbic acid). The MDHAA-AA in this band (R_F 0.12) reacted slowly with 2,6-dichlorophenolindophenol at room temperature. Good reaction with the spray reagent was obtained by heating the chromatograms at 100° for 10 minutes. Dilute aqueous solutions of MDHAA-AA taken to just short of dryness and rechromatographed showed a single band, R_F 0.12 (solvent system B), with only a slight trace of material at R_F 0.37. A 10-minute exposure of this solution to hydrogen sulfide followed by rechromatography resulted in one band at R_F 0.37. In the solid state the MDHAA-AA appeared as yellow needles (mp 281–284° decomp; λ_{max}^{water} = 265, *p*H 7). Attempts at elemental analysis were unsuccessful because of the extreme hygroscopic nature of the compound. The neutralization equivalent was 330 (±5%).

The MDHAA-AA complex was found to be soluble in water, methanol, and ethanol, slightly soluble in ether, and insoluble in most organic solvents. A concentrated solution of MDHAA-AA gave a blue test with *N,N'*-diphenylbenzidine. Spraying a chromatogram of the air-oxidized solution with iodine gave an area of decolorization at R_F 0.12 and 0.37 (solvent system B).

A 2,4-dinitrophenylhydrazine (DNPH) derivative was prepared by making the aqueous solution of MDHAA-AA 3 N with respect to HCl, and adding a 4:1 mole ratio of DNPH in HCl. It is essential that all reagents used in derivative formation be pure. The DNPH must be recrystallized to a melting point of 198–200°. The derivative, which precipitated immediately upon addition of DNPH, was collected and washed extensively with water until the ash water was colorless, then recrystallized from acetone-alcohol (1:1) and from carbon tetrachloride. The crystals appeared as pale yellow plates (mp 119–121°; $\lambda_{max}^{CCl_4}$ = 348 (ϵ = 7488); C, 45.8%; H, 4.14%; N, 21.6%).

A chromatographically pure sample of 2,3-diketogulonic acid, R_F 0.16 (solvent system B), from an air-oxidized solution of ascorbic acid was allowed to react with DNPH as before. The derivative precipitated in 1 hour at room temperature. The crystals appeared as bright orange needles (mp 119–120.5°; $\lambda_{max}^{CCl_4}$ = 348 (ϵ = 7090); C, 45.1%; H, 4.1%; N, 21.0%).

A DNPH derivative was prepared of ascorbic acid as above. The derivative precipitated in 12 hours at room temperature. The crystals appeared as pale yellow plates (mp 119–121°; $\lambda_{max}^{CCl_4}$ = 348 (ϵ = 7347); C, 45.0%; H, 4.1%; N, 22.8%).

The mixture melting points of the ascorbic acid derivative and that of the MDHAA-AA derivative showed no depression. The mixture melting points of the 2,3-diketogulonic acid derivative and that of

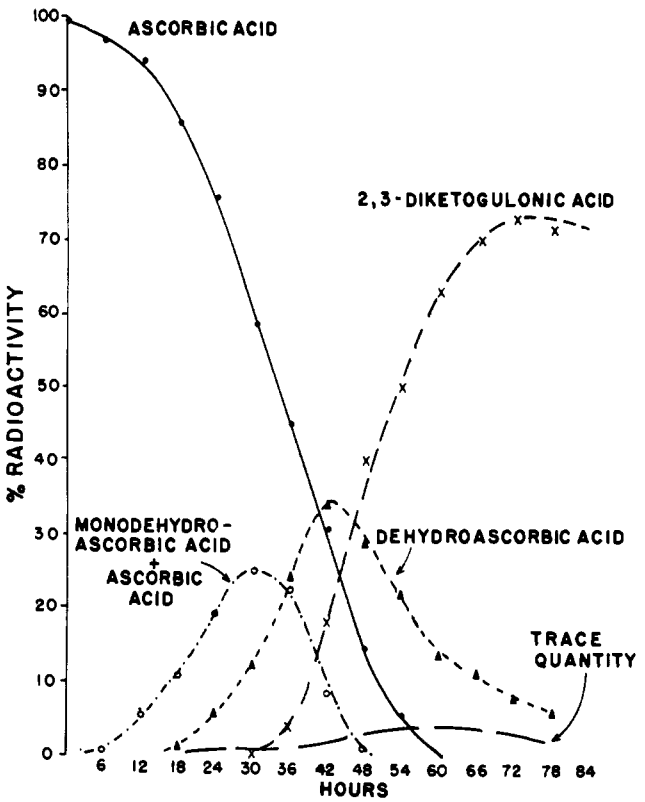


FIG. 3.—Degradation of L-ascorbic acid in solution as a function of time at 37°.

ascorbic acid showed a depression to 117–119°, and that of 2,3-diketogulonic acid and MDHAA-AA showed a depression to 117.5–119°.

The three derivatives were shown to be chromatographically pure. The infrared spectra (CCl_4, 0.1%) are shown in Figure 1, and indicate that the MDHAA-AA derivative and the ascorbic acid derivative are similar but not identical, and do possess a lactone ring which is not seen in the derivative of 2,3-diketogulonic acid.

Changes in *p*H were also discernible (Beckman expanded scale *p*H meter) during the autoxidation of the aqueous L-ascorbic acid solution (Table I). The *p*H reached a maximum after 36 hours, then dropped to 2.65 after 72 hours. Changes in optical rotation were also seen during the autoxidation. A 1% solution of freshly prepared ascorbate had a specific rotation of 21.0° in water (Table I). The specific rotation reached a maximum of 57.5° after 72 hours, then decreased steadily. (Solutions used in the *p*H and specific-rotation studies were maintained at 37° ± 1°.)

TABLE I

SPECIFIC ROTATION, $[\alpha]_D^{25}$, AND *p*H OF L-ASCORBIC ACID SOLUTION AS A FUNCTION OF TIME[a]

Time (hr)	Specific Rotation	*p*H
0	+20.5°	2.71
12	+30.0°	2.76
24	+35.0°	2.79
36	+43.0°	2.82
48	+50.1°	2.73
60	+55.0°	2.69
72	+57.5°	2.65

[a] Solution (1%) stored at 37°.

A freshly prepared solution of L-ascorbic acid had a specific rotation of 21.0° (*p*H 2.71). At a *p*H of 4.21 the specific rotation had changed to 43.2°, and at neutrality the specific rotation was 119°.

Kinetic studies using aqueous L-[1-^{14}C]ascorbic acid, stored at 25° ± 1° and 37° ± 1°, were serially chromatographed (solvent system B) at 6-hour intervals through 96 hours. The autoradiograms prepared from these chromatograms are shown in Figure 2a,b. The chromatograms were then cut into strips and scanned on a 4π integrator scanner, and the per cent activity per band per unit time was calculated (Fig. 3).

DISCUSSION

Chromatography of air-oxidized ascorbic acid solutions yielded R_F values which correlated with the known R_F values for ascorbic acid and its oxidation products except in the location of bands at R_F 0.09 and 0.12 (solvent system A) and 0.12 and 0.16 (Kamiya, 1960b) (solvent system B). All samples of ascorbic acid gave similar chromatograms, which were confirmed with the use of the ^{14}C-labeled ascorbate.

The first-order characteristics of the reaction are apparent from Figure 3, as is the typical autoxidative curve of the decrease of total ascorbate. When 1% aqueous solutions of ascorbate were prepared with 1 g hydroquinone per 100 ml of solution, a decrease in the rate of reaction was apparent. When thioctic acid was added (50 mg/100 ml) to freshly prepared aqueous solution of ascorbate, a decrease (33%) in the rate of reaction was observed. In rate studies it was found that when L-[1-^{14}C]ascorbic acid was isolated, and unlabeled MDHAA-AA was added to a freshly dissolved solution of the labeled ascorbate, the rate of intermediate formation was much greater than the original rates. This was consistent with the proposed radical mechanism of the reaction and the autocatalytic role of the complex.

The autoradiograms (Fig. 2) show a significant amount of radioactivity at the point of application in the chromatograms of samples taken at 24, 30, 36, and 42 hours. The only substance that could result from the oxidation, maintain radioactivity, and not migrate in the solvent system is oxalic acid. This compound, labeled as trace quantity in Figure 3, was found not to exceed 5% of the total activity, during the kinetic radiochromatographic study.

During the 72-hour counting period used to determine the specific activity of the sample of L-ascorbic acid, a consistent decrease in total activity was seen. Only a loss of $^{14}CO_2$ could account for this decrease. Another sample was prepared and the $^{14}CO_2$ was trapped in hyamine and counted. The activity of this $^{14}CO_2$ accounted for 25–28% of the total activity of the sample.

Although both hydroxyls of the ene-diol system of ascorbic acid are acidic, the first dissociation constant ($pK_1 = 4.1$) is associated with the hydroxyl on the third carbon of the lactone ring, and the second dissociation constant ($pK_2 = 11.6$) is associated with the hydroxyl at carbon 2 of the ring. During the autoxidation, changes in *p*H were observed. These changes did not exceed pK_1 of the acid and must be owing to changes involving the third carbon since the molecule itself remains intact. Bonding in the complex must then be across the third carbon of the lactone ring inhibiting keto-enolization of this area of the molecule. Titration of the solution of the complex to a *p*H 8 end point then supports the contention that only one equivalent of alkali is required in the determination of the neutralization equivalent, and an approximate molecular weight of 330 is obtained.

The exact nature of bonding in the monodehydro-

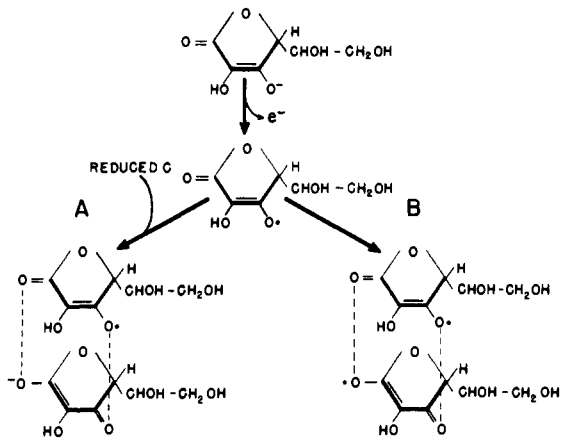


FIG. 4.—Proposed oxidative pathway to monodehydroascorbic acid and MDHAA-AA complex.

ascorbic acid–ascorbic acid complex is still questionable. A possible complex is shown in structure A, Figure 4. This would give derivative formation found under the conditions used in these experiments, and as here supported by the reaction with *N,N'*-diphenylbenzidine and iodine. This structure would also show the titratable hydrogen observed. The formation of charge-transfer complexes are well known, and this may be the type functioning in MDHAA-AA.

The existence of a monodehydroascorbate dimer, as shown in structure B, Figure 4, was first postulated in a modified form by Bezssonoff and Woloszyn (1938). Such a structure, while analogous to some of the intermediates observed in the oxidation of the hydroquinones, is inconsistent with the chemical properties of our monodehydro intermediate.

The possibility that the intermediate (R_F 0.12, solvent system B) was the dimeric (Fig. 5) form of dehydroascorbic acid (Kenyon and Munro, 1948; Pecherer, 1951) and not MDHAA-AA was investigated. This dimeric form of dehydroascorbic acid was prepared and the DNPH derivative was found to be quite different in its rate of formation, melting point, appearance, and infrared-band absorption, from that of MDHAA-AA and its DNPH derivative.

The possibility of organic sulfur compounds forming during the chromatographic run was considered; tests were performed on the isolated MDHAA-AA complex, and indicated no sulfur in the compound tested. The fact that both labeled oxalate and labeled $^{14}CO_2$ were generated during the autoxidation of L-ascorbic acid implies that the oxidative pathway is taking two courses, the predominant reaction being the decarboxylation of dehydroascorbic acid and 2,3-diketogulonic acid to L-xylonic acid and L-lyxonic acid (Kagawa, 1962).

CONCLUSIONS

The studies in this laboratory have effected the isolation and characterization of an intermediate compound in the autoxidative pathway of L-ascorbic acid to dehydroascorbic acid. This intermediate is monodehydroascorbic acid and may be complexed with ascorbic acid. Aqueous solutions of ascorbic acid and [1-^{14}C]ascorbic acid, which develop a yellow color on

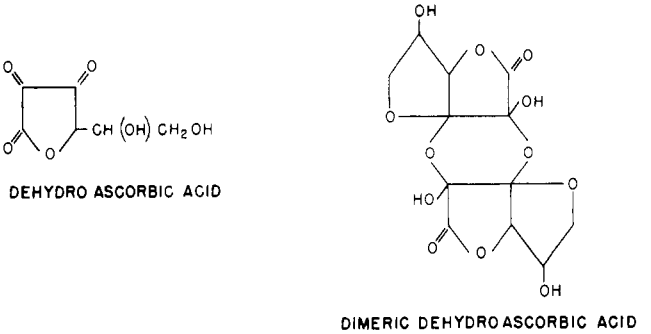


FIG. 5.—Structural formulas of dehydroascorbic acid and dimeric dehydroascorbic acid.

standing, were chromatographed. In the solvent system of Tegethoff (1953) the R_F of the complex is 0.12. The compound appears as yellow needles (mp 284° decomp., λ_{max}^{water} = 265, *p*H 7).

The use of ^{14}C-labeled ascorbate positions the intermediate in the autoxidative pathway and verifies its existence as a product of this process by kinetic radiochromatographic studies. Close similarity of the ultraviolet spectra of the intermediate and the ascorbic acid indicates stability of the functional groups. The neutralization equivalent suggests that the intermediate complex has an intact lactone ring and an approximate molecular weight of 330.

The gradual decolorization of paper-chromatograph spots developed with 2,6-dichlorophenolindophenol gives evidence for a reversible complexing of some oxidized form of ascorbic acid with reduced ascorbic acid. During the autoxidation, the *p*H changes show that the third carbon is involved in forming the monodehydroascorbic acid–ascorbic acid complex.

Reaction rate studies show that there is an autocatalytic action of monodehydroascorbic acid–ascorbic acid complex in the autoxidative process.

REFERENCES

Baker, E. M., Levandoski, N. G., and Sauberlich, H. E. (1963), *Proc. Soc. Exptl. Biol. Med. 113*, 379.
Barr, N. F., and King, C. G. (1956), *J. Am. Chem. Soc. 78*, 303.
Bezssonoff, M. N., and Woloszyn, W. (1938), *Bull. Soc. Chim. Biol. 20*, 93.
Campbell, J., and Tubb, W. G. (1950), *Can. J. Research* (now *Can. J. Biochem. Physiol.*) *28E*, 19.
Edsall, J. T., and Sagall, E. L. (1943), *J. Am. Chem. Soc. 65*, 1312.
Finholt, P., Paulssen, R. B., and Higuchi, T. (1963), *J. Pharm. Sci. 52*, 948.
Géro, E., and Le Gallic, P. (1952), *Compt. Rend. 234*, 145.
Herrmann, J., and Andrae, W. (1963), *Nahrung 7*, 411.
Kagawa, Y. (1962), *J. Biochem. (Tokyo) 51*, 134.
Kamiya, S. (1960a), *Nippon Nogeikagaku Kaishi 34*, 8.
Kamiya, S. (1960b), *J. Vitaminol. (Kyoto) 6*, 217.
Kenyon, J., and Munro, N. (1948), *J. Chem. Soc.*, 158.
Levandoski, N. G., Baker, E. M., and Stickler, W. C. (1963), *Federation Proc. 22(2)*, 437 (abstract).
Mapson, L. W., and Partridge, S. M. (1949), *Nature 164*, 479.
Nace, H. R., and King, K. F. (1962), *J. Med. Pharm. Chem. 5*, 1316.
Pecherer, B. (1951), *J. Am. Chem. Soc. 73*, 3827.
Tegethoff, B. (1953), *Z. Naturforsch 8b*, 374.