Acknowledgment

The authors gratefully acknowledge the cooperation and helpful suggestions of a number of companies which supplied the samples of floccing and nonfloccing sugars used in various phases of this investigation. These include: The Coca-Cola Co., Atlanta, Ga.; Reserve Division, National Sugar Refining Co., New Orleans, La. (Formerly Godchaux Sugars, Inc.); Refined Syrups and Sugars, Inc., Yonkers, N. Y. Thanks are due the Amalgamated Sugar Co., Twin Falls, Idaho, for a sample of sugar beet saponin.

The authors are also indebted to T. H. Hopper for direction and guidance; and to L. E. Brown, Alva F. Cucullu, Julian F. Jurgens, S. M. Stark, Jr., James A. Harris, Dorothy C. Heinzelman, Donald Mitcham, J. W. Dieckert, R. L. Ory, and Nelle J. Morris for technical assistance and helpful suggestions.

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Received for review September 15, 1958. Accepted February 2, 1959. Trade names are given as part of the exact experimental conditions and not as an endorsement of the products.

FOOD ANTIOXIDANTS

Biochemistry of Erythorbic Acid. Human Blood Levels and Urinary Excretion of Ascorbic and Erythorbic Acids

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Recent widespread use of erythorbic acid as an antioxidant in foods raises the question of masking of true L-ascorbic acid levels in assessment of nutriture. The usual methods for determining ascorbic acid in blood do not distinguish between these acids. Comparative blood level trials in five normal subjects showed that the ingestion of erythorbic acid which has less than 1% of the vitamin C activity of L-ascorbic in animals, can lead to overestimation of the state of vitamin C nutrition as judged by blood levels. A rapid, quantitative paper chromatographic technique for separation of the isomers showed no significant displacement of L-ascorbic by 300-mg. doses of erythorbic acid.

ERYTHORBIC ACID, also known as D-araboascorbic or D-isoascorbic acid, a stereoisomer of L-ascorbic acid (Figure 1), has been widely used in foods in recent years as the antioxidant properties of this more easily synthesized isomer are similar (*2*) to those of L-ascorbic acid. However, marked differences have been reported in the chemical, physical, and biological properties of the two isomers as summarized in Table I. In guinea pigs, erythorbic acid was claimed to be only about 5% as potent as L-ascorbic acid with respect to antiscorbutic activity (*14*) and to effects on serum phosphatase (*3*) and excretion of intermediary tyrosine metabolites (*11*). This 1 to 20 relationship does not hold for the marked differences in reactivity of the isomers with ascorbic acid oxidases (*7*) and reductases (*4, 13*).

Recent guinea pig tests (*9*) have shown the activity of erythorbic acid to be due to its sporing action on L-ascorbic acid. In severely depleted animals, erythorbic acid has less than 1% of the antiscorbutic activity of L-ascorbic acid. Attempts to demonstrate antagonism between the isomers were unsuccessful in serum phosphatase tests in guinea pigs (*3*), but complete inhibition by erythorbic acid of an ascorbic acid oxidation enzyme in a fungus has been demonstrated (*7*). Ikeuchi (*5*) reported urinary excretion of erythorbic and ascorbic acid after dosage to vitamin C-deficient humans,

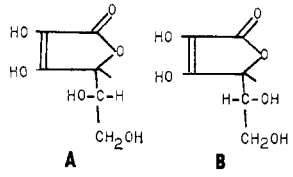

Figure 1. Stereochemical configurations

A. L-Ascorbic acid (vitamin C)
B. Erythorbic acid

Table I. Physical, Chemical, and Biological Differences

	L-Ascorbic Acid	Erythorbic Acid
Specific rotation	+20.5° to 21.5°	−16.7° to −17.7°
Melting point	190–192° C.	164–168° C.
Nicotinic acid addition compound	+	−
Uranium complex in alkaline solution	Unstable	Stable
Antiscorbutic activity in guinea pigs	1	0 to 1/20
Effect of serum phosphatase in guinea pigs	1	1/20
Preventing excretion of intermediate tyrosine metabolites in guinea pigs	1	1/20
Rate of oxidation by ascorbic acid oxidases	1	0 to 2/25
Rate of reduction of dehydro forms by reductase systems	1	1/3 to 1/2

which indicated little, if any, biochemical utilization of erythorbic acid.

As blood levels are normally used in assessing the state of a person's vitamin C nutrition and as commonly used clinical methods for determining blood levels of ascorbic acid will also measure erythorbic acid, the appearance of significant amounts of the latter in the blood following ingestion of this isomer would give a distorted picture of the status of an individual with regard to vitamin C nutriture. Hence, blood levels of erythorbic acid after oral dosage to humans were studied.

In addition, measurements were made of urinary excretions of L-ascorbic acid after oral doses of erythorbic acid to determine whether this less active isomer could displace L-ascorbic acid from the tissues of saturated subjects. This part of the study required an investigation of published methods for separating ascorbic from erythorbic acid by paper chromatography. A procedure was developed for quantitative colorimetric estimation of the two isomers in urine.

Experimental

Blood Studies. Blood analyses were done by the micromethod of Lowry and coworkers (*6*), a procedure commonly used for nutrition surveys. In this procedure, cupric ion converts the isomers to their dehydro forms which are then coupled with 2,4-dinitrophenylhydrazine at 37° C. followed by colorization of the coupled forms with 65% sulfuric acid. Minor deviations from this procedure include the use of whole blood instead of serum and performing the cupric ion conversion and dinitrophenylhydrazine coupling in 16 hours at room temperature instead of in 4 hours at 37° C. Use of whole blood eliminates the necessity for the considerable manipulation of blood collecting tubes and involves direct transfer of the blood to test tubes containing the protein precipitant. Furthermore, whole blood levels are considered a better index of vitamin C nutrition than serum or plasma levels (*12*).

Urinary Studies. Urinary excretion studies to test the possibility of displacement of L-ascorbic acid by erythorbic acid were performed on subjects saturated with L-ascorbic acid. To ensure saturation, the subjects were dosed orally with 500 mg. of L-ascorbic acid per day for a period of at least 7 days. Tests were run only on those subjects who, the day before, had excreted at least 40% of the 500-mg. saturating dose in the 4 hours following dosage. The 4-hour basal excretions were measured by omitting the daily 500-mg. saturation dose on that particular day. Saturation doses were again given orally until the day before the following urine collection period. Urinary levels of L-ascorbic and erythorbic acids were measured in a parallel 4-hour period after oral dosage of 300 mg. of erythrobic acid given either as a single dose or in 50- to 100-mg. increments at 15- to 30-minute intervals. Saturation doses were given through the day just preceding the test day with the time schedule exactly as followed for the basal excretion period.

Paper chromatographic separations of ascorbic and erythorbic acids in urine were made by a modification of the qualitative method of Mitchell and Patterson (*8*). The modifications, which were designed to make the separation quantitative, more rapid and applicable to urine, included use of 3-hour descending chromatography instead of the 6-hour ascending technique and prior impregnation of the paper with 2% instead of 1% metaphosphoric acid to stabilize the isomers during chromatography. The higher concentration of metaphosphoric acid is necessary to give a successful separation when a relatively large volume of urine is applied per spot. The 2% solution is prepared on the day of use. The best separation of the isomers was obtained by application per spot of 20 to 40 λ containing 10 to 20 γ of each acid. Spotting and chromatographic development in daylight led to losses of 13 to 17%, which were eliminated by spotting under Wratten OA safelights and developing the chromatograms in an unlighted room with the shades drawn.

Quantitation was accomplished by using a guide chromatogram alongside three test chromatograms, the whole consisting of four spots applied evenly along a 6 × 22½ inch sheet of Whatman No. 1 paper. After being spotted, the paper was placed in a 12 × 24 inch cylindrical chromatography jar containing about 50 ml. of the mobile solvent. This consists of a mixture of acetonitrile, acetone, water, and glacial acetic acid in proportions by volume of 80:5:15:1.2, respectively. About 1.5 hours were allowed for saturation of the sealed system before the mobile solvent mixture was added to the chromatographic troughs. After development, during which the solvent fronts approached to within an inch of the bottom, the papers were removed and dried for about 15 minutes in a ventilated hood.

The guide chromatogram was then separated by cutting lengthwise and dipped in a 0.03% aqueous solution of 2,6-dichlorophenolindophenol. White spots against a pink background indicate the exact location of the isomers. Dipping the guide chromatogram, instead of spraying as recommended in the methods investigated for separation of these isomers (*1, 8*), yielded sharper delineation of spots. The 0.03% concentration of dye is suitable for the amounts of reducing isomers in the present tests. If more or less ascorbic or erythorbic acid is present, the dye concentration can be varied from 0.05% to 0.005% as needed. The corresponding areas on the test chromatograms were cut out transversely.

As part of the reagent blanks, strips of paper of similar area were cut out below the erythorbic acid and above the ascorbic acid spots. Separate estimates of L-ascorbic and erythorbic acids and their respective blanks were made by thorough agitation of the folded paper strips in aqueous metaphosphoric acid with a rod followed by addition of the colorimetric reagents to the same vessel without removing the disintegrated strips.

Colorimetry was by the indophenol-xylene extraction technique (*10*). While the R_f values of the two isomers are fairly close, 0.38 and 0.43 for L-ascorbic and erythorbic acids, respectively, separation was surprisingly successful starting with an oval spot as large as ¼ × ½ inch on which as much as 100 λ (0.1 ml.) of urine had been applied slowly while drying with warm nitrogen. The use of these large spots eliminated the necessity for a preparative isolation of the isomers by paper chromatography

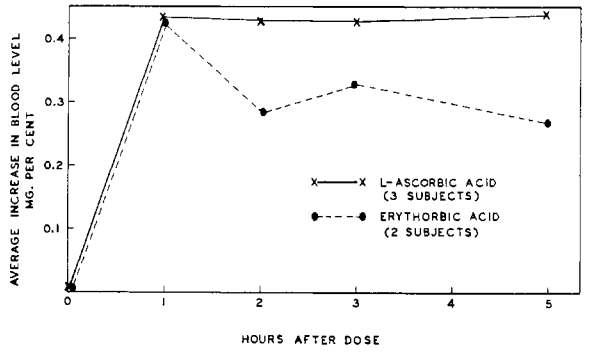

Figure 2. Whole blood levels of L-ascorbic or erythorbic acid in humans after a 500-mg. oral dose

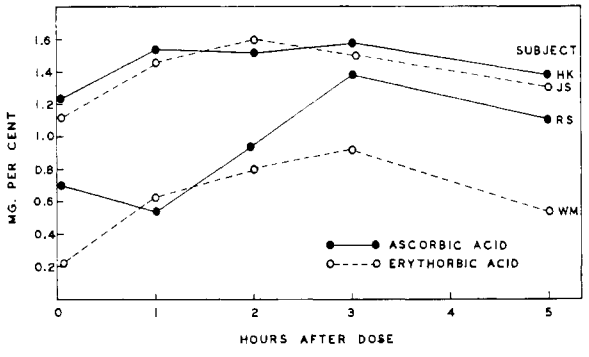

Figure 3. Average increase of whole blood levels of L-ascorbic or erythorbic acid in ascorbic acid-saturated humans after a 200-mg. oral dose

followed by a tedious and time-consuming extraction and concentration prior to a second stage paper chromatographic separation as practiced for urine and other biological extracts by Chen, Isherwood, and Mapson (*7*).

Little or no interference from other reducing substances was indicated by recoveries of 101 and 108%, respectively, for L-ascorbic and erythorbic acids added to a representative urine specimen and chromatographed by this procedure. A ratio of isomers of 5 to 1 was easily separable. Four urine specimens can be processed in an 8-hour period. The relatively short handling period minimizes losses due to the well-known instability of these isomers.

Results and Discussion

Following oral dosage to humans at the 500-mg. level, the blood level curves (Figure 2) with the two isomers are closely comparable. Subjects H. K. and J. S., whose predosage levels indicated saturation, show an almost identical rate of rise and fall on 500-mg. dosage of L-ascorbic and erythorbic acid, respectively. Subject W. M., having a basal level apparently in the deficiency range, responds to erythorbic acid dosage so that his blood levels are elevated to the vitamin C–sufficient level. As in this first experiment the doses were large and some of the subjects apparently not saturated, for the second experiment the subjects were saturated with L-ascorbic acid and the dosage was lowered to 200 mg. (Figure 3). The increases above the predose levels are closely comparable for both isomers and are maintained for at least 5 hours. Ingestion of foods to which erythorbic acid is added simply as an antioxidant could lead to an overestimation via blood level tests of the state of vitamin C nutrition.

The results of the urinary excretion tests in Table II indicate no significant displacement of L-ascorbic acid by the 300-mg. dosage of erythorbic acid. The average excretion of L-ascorbic acid is practically identical to the average basal excretion. Zilva (*15*) has reported that a slight deposition of erythorbic acid—consistent with its antiscorbutic activity—occurs in the tissues of vitamin C–deficient guinea pigs. If erythorbic acid had significant antiscorbutic activity in humans, this isomer would be expected to be deposited in human tissues and thus displace L-ascorbic acid from saturated subjects As the present study indicates that there is no significant displacement of L-ascorbic acid by erythorbic acid, it suggests different metabolic pathways for the two isomers and appears to confirm Ikeuchi's conclusion (*5*) that the antiscorbutic activity of erythorbic acid in humans is trifling compared to that of L-ascorbic acid.

Table II. Effect of 300-Mg. Oral Dose of Erythorbic Acid on Human Urinary Excretion of L-Ascorbic Acid

	Excretion in 4 Hr. after Dose, Mg.	
Subject	Basal	Post dosage
H.K.	27	22
W.M.	40	43
M.O.	38	39
J.S.	27	31
J.V.	26	25
	Av. 31.6	32.0

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Received for review October 15, 1958. Accepted February 4, 1959. Presented before the American Institute of Nutrition, Federation of American Societies for Experimental Biology, Philadelphia, Pa., April 1958.