Spectrophotometric method for determination of tocopherol in red blood cells

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Abstract A relatively rapid procedure is described for the spectrophotometric determination of total tocopherol in red blood cells (RBC) based on a modification of the original Emmerie-Engel reaction. The critical feature in this method is the presence of a large amount of an added antioxidant, pyrogallol or ascorbic acid, during the saponification and extraction stages and the use of thin-layer chromatography for tocopherol purification. The total tocopherol levels of plasma and erythrocytes were determined for a number of human subjects, for patients with abetalipoproteinemia, and for rats. It was found that these levels had a wide range in normal human subjects but that the ratio of RBC to plasma tocopherol was relatively constant and equal to 0.18, uncorrected, and 0.21 when both RBC and plasma values were corrected to 100% recovery. The RBC-to-plasma ratio for rats was 0.39. The accuracy of this ratio determined by the spectrophotometric procedure was verified by measuring the distribution of [^{14}C]tocopherol in RBC and plasma when radioactive vitamin E was introduced into the blood by both in vitro and in vivo techniques. The addition of radioactive tocopherol to RBC or plasma at the initial stage of the analysis permits an accurate determination of the total tocopherol in RBC or plasma by calculations based on the recovery of the added isotope. This procedure for erythrocyte tocopherol analysis is compared with a gas–liquid chromatographic method in current use.

Supplementary key words α-tocopherol · vitamin E · erythrocyte α-tocopherol · abetalipoproteinemia

Vitamin E is a widely distributed fat-soluble vitamin composed of several tocopherols and tocotrienols, the most biologically active of which is α-tocopherol. The terms vitamin E and tocopherol are commonly used interchangeably, as they are in this paper. The precise role that vitamin E plays in human metabolism is still in dispute, but there is general consensus that it is important for erythrocyte stability. The great majority of studies concerned with erythrocyte stability and tocopherol levels have used hemolysis of the erythrocyte by hydrogen peroxide as an end point, rather than the analytical measurement of red cell tocopherol (1, 2). This roundabout biological determination has been used because no satisfactory method for measuring tocopherol in erythrocytes was available.

We have been interested in tocopherol metabolism and transport in human plasma and erythrocytes for a number of years. Patients with abetalipoproteinemia who were not receiving tocopherol supplementation in their diet had increased susceptibility of their red cells to hemolysis by hydrogen peroxide (3). This abnormality was corrected by oral administration of a vitamin E preparation, α-tocopherol polyethylene glycol succinate (4). The original problems in measuring tocopherol in the erythrocytes (5) led to further studies of several analytical techniques; the method presented here for analyzing tocopherol in red blood cells is the result of these investigations. The procedure is based upon the original Emmerie-Engel tocopherol assay (6); the critical step in our modification is the addition, at the initial saponification step, of a very large amount of an antioxidant, ascorbic acid or pyrogallol. The colorimetric reaction is made more sensitive by the substitution of bathophenanthroline for 2,2′-bipyridine in the reaction with ferrous ion.

In earlier studies the tocopherol levels in animal erythrocytes were estimated indirectly after radioactive α-tocopherol was fed to rats and the distribution of the labeled vitamin in the RBC and in plasma was determined (7–9). The tocopherol levels of rat and rabbit erythrocytes have been estimated by paper chromatography and colorimetric analysis (10, 11). Finally, a gas–liquid chromatographic method has been developed by Bieri, Poukka, and Prival for assay of tocopherol in erythrocytes and has been used in both animal and human studies (12).

EXPERIMENTAL PROCEDURES

Materials

All solvents were reagent grade and redistilled prior to use. The bathophenanthroline used as the color reagent

Abbreviations: TLC, thin-layer chromatography.

was purchased from G. Frederick Smith Chemical Co., Columbus, Ohio. DL-α-Tocopherol and the acetate ester were generously provided by Hoffmann-La Roche, Inc., Nutley, N.J. α-Tocopherylquinone was purchased from General Biochemicals, Chagrin Falls, Ohio. DL-α-[3,4-^{14}C]Tocopherol was prepared from DL-α-[^{14}C]tocopheryl acetate via mild KOH hydrolysis in ethanol followed by addition of water, extraction of the tocopherol with hexane, and purification by TLC on silica gel G using benzene–ethyl acetate 2:1 as the solvent system. The [^{14}C]tocopheryl acetate was supplied by Hoffmann-La Roche, Basle, Switzerland. Specific activity was 2.98×10^4 dpm/μg.

TLC was carried out on precoated and prescored silica gel G plates purchased from Analtech, Inc., Newark, Del. The plates were activated at 110°C for at least 1 hr prior to use. The solvent system used for separating tocopherol, tocopherylquinone, and cholesterol was benzene–ethyl acetate 2:1. An alternative and preferred solvent system is benzene–ethanol 99:1. The chromatography tank was flushed with nitrogen before use and the separations were carried out away from direct sunlight. Spectrophotometry was carried out using a Gilford model 240 equipped with an automatic sampler.

Radioactive samples were counted on a Packard Tri-Carb scintillation spectrometer 3375 using Liquifluor (New England Nuclear Corp., Boston, Mass.) as the scintillation solvent. Where aqueous samples or samples containing protein, etc., were involved, Scintisol-GP (Isolab, Inc., Elkhart, Ind.) was added to the standard toluene cocktail in a ratio of 2 ml of Scintisol to 10 ml of toluene fluor.

ANALYTICAL METHOD

Red blood cells

Fresh venous blood was transferred directly from a plastic syringe into two 50-cc plastic round-bottomed centrifuge tubes containing 0.2 ml of 1% Na_2EDTA/10 ml of whole blood. The blood and EDTA were mixed immediately by gently inverting the tubes three or four times. The blood was then centrifuged in a Sorval GLC-1 centrifuge at 2000 rpm (about 500 g), and the plasma and buffy layer were removed by aspiration. The red blood cells were washed three times with 5 vol of an isotonic phosphate-buffered NaCl solution containing EDTA. The buffer consisted of 1.42 g of anhydrous Na_2HPO_4, 7.27 g of NaCl, and 0.1 g of Na_2EDTA made to 1 l with distilled water and adjusted to pH 7.4 with dilute HCl. The final hematocrit of the RBC after the third washing was made up to about 50% and accurately measured in a standard Adams microhematocrit centrifuge.

Saponification and extraction

2 ml of washed red blood cells with a known hematocrit and red cell count were pipetted into 50-ml glass-stoppered centrifuge tubes. 10 ml of a 2% solution of pyrogallol in ethanol was slowly added while mixing; after thorough mixing, the tubes were loosely stoppered and heated for 2 min in a 70°C water bath. The tubes were removed from the hot water bath, and after addition of 0.5 ml of saturated KOH the tubes were heated at 70°C for 30 min. After saponification, the samples were cooled in an ice bath.

The saponified material was extracted at room temperature after addition of 7.5 ml of distilled water and then 22 ml of hexane. The tubes were then tightly stoppered and shaken vigorously by hand for 2 min. The tubes were centrifuged at approximately 1500 rpm to ensure complete phase separation.

Thin-layer chromatography

20 ml of the hexane phase (upper layer) was removed by pipette and evaporated under nitrogen. A small amount of chloroform (20–50 μl) was added to the tubes to dissolve the residue, and this solution was spotted on silica gel G plates (the lanes were 2 cm wide). Pure α-tocopherol was also spotted as a standard, and the plate was developed in a solvent system of benzene–ethyl acetate 2:1, or, preferably, in a solvent system of benzene–ethanol 99:1. After development, the tocopherol band was located by spraying the plate with methanolic 0.001% rhodamine 6G and examining it under UV light. (It is to be noted that prolonged exposure to UV light decomposes the tocopherol molecule.) The area of silica gel corresponding in R_F to the tocopherol standard was scraped from the plate with a razor blade into a 7-ml centrifuge tube. For eluting the tocopherol from the silica gel 1.5 ml of 100% ethanol was added, and the contents were thoroughly mixed with a Vortex mixer; the tubes were then centrifuged at 2500 rpm for 5 min.

Spectrophotometric analysis

1 ml of the ethanol solution was pipetted into a 4-ml glass test tube for analysis. Standards of 1, 2, 5, and 10 μg of α-tocopherol were made up to 1 ml in 100% ethanol at the same time. 0.2 ml of 0.2% bathophenanthroline in ethanol was added and the contents of each tube were thoroughly mixed. The assay proceeded rapidly from this point and care was taken not to expose the solutions to direct light. 0.2 ml of a 0.001 M $FeCl_3$ solution in ethanol was added, followed by mixing with a Vortex mixer. After 1 min, 0.2 ml of 0.001 M H_3PO_4 solution in ethanol was added and the contents of the tubes were again thoroughly mixed. The absorbance of the solutions was

determined at 534 nm using an automatic sampler and a Gilford model 240 spectrophotometer.

Plasma or serum tocopherol assay

The procedure used for the determination of tocopherol levels in plasma or serum was basically the same as that just described for the erythrocytes. 1 ml of plasma was pipetted into a glass-stoppered 15-ml centrifuge tube and 2 ml of a 2% solution of pyrogallol in ethanol was added while mixing. This solution was heated at 70°C for 2 min after which 0.3 ml of saturated KOH was added. After thorough mixing, this mixture was placed in the 70°C water bath for 30 min. The tubes were cooled in ice and 1 ml of water and 4 ml of hexane were added. The contents were shaken vigorously by hand for 2 min and centrifuged to completely separate the phases. 3 ml of the hexane phase (upper layer) was pipetted into another centrifuge tube and evaporated under nitrogen. The remainder of the analysis followed the RBC procedure from the TLC step.

In vitro incubations

25-ml Erlenmeyer flasks were silanized by addition of 5 ml of a 10% solution of hexamethyldisilazane (Applied Science Laboratories) in toluene. After shaking the flasks for 1–2 hr in a 37°C water bath, they were rinsed three times (acetone, water, acetone) and dried under a stream of nitrogen. α-[^{14}C]Tocopherol (0.13 μCi and 10 μg) was dissolved in 0.3–0.5 ml of benzene. This solution was added to the flask and then evaporated under nitrogen while rotating the flask by hand to disperse the vitamin as evenly as possible on the glass. Precautions were taken for minimum exposure of these coated flasks to air and light since the tocopherol at this concentration is very susceptible to oxidation.

Fresh EDTA–blood (human or rat) was added immediately to the [^{14}C]tocopherol-coated flask, and the incubation was carried out for 4–6 hr at 37°C with gentle agitation. The blood was then transferred to another silanized flask not containing any tocopherol and incubated 2–4 hr at 37°C to ensure equilibration of [^{14}C]tocopherol. This additional incubation was not crucial for blood from normal human subjects since the ratio of radioactivity in RBC to that in plasma after the initial incubation was very close to that observed at the end of the final incubation. The RBC were then separated by moderate centrifugation and washed three times with 5 vol of buffer. Both the RBC and the plasma were saponified and extracted as described above.

Feeding α-[^{14}C]tocopherol to rats

For both the feeding and incubation studies in rats, female Sprague-Dawley animals weighing 300–350 g were used. An ethanolic solution of the radioactive vitamin E (20 μCi) was evaporated under nitrogen to about 50 μl and then added to 2 ml of a 50% mixture of olive oil and oleic acid. Each animal was fed 0.5 ml of this mixture with a feeding needle and then fasted overnight. The following day the animals were anesthetized with ether, the abdomen was opened, and blood was withdrawn from the aorta and collected in EDTA. The RBC and plasma were then separated in the usual way.

Calculations

The following equation was used for the calculation of the concentration of tocopherol (μg/ml) in packed red blood cells.

$$\frac{[(A/B \times 22/20 \times 1.5/1.0) \times 100/C]}{D} - Z = Y$$

where A is the absorbance of the sample read from the spectrophotometer, B is the absorbance of 1 μg of standard tocopherol, C is the percentage recovery of α-[^{14}C]tocopherol added at the beginning of the procedure, D is the volume (ml) of packed RBC found by multiplying the volume of washed RBCs analyzed by the hematocrit, Y is μg of tocopherol per ml of packed RBC corrected to 100% recovery of tocopherol, and Z is the mass of the [^{14}C]tocopherol added per ml of packed RBC.

The factor 22/20 arises from the first hexane extraction where 20 ml is used for analysis out of the original 22 ml added. The factor 1.5/1.0 arises from the ethanol elution of the silica gel where 1.0 ml is used for analysis out of the original 1.5 ml added.

When no radioactive tocopherol is added, the equation becomes simplified since the terms containing C and Z are omitted. For the highest possible accuracy, it is recommended that radioactive tocopherol be added to the original plasma and red blood cell samples before analysis. However, we have found that the method is accurate and adequately reproducible so that for routine analysis, where equipment for measuring radioactivity is not available, the method may be used without the isotope recovery. Corrections for recovery in our laboratory average 15% for plasma and 30% for red blood cells. Duplicate samples are always analyzed, and the variation between duplicates does not exceed 5% for plasma and 10% for red blood cells. In these studies the [^{14}C]tocopherol had a specific activity of 2.98×10^4 dpm/μg. The radioactive tocopherol was dissolved in absolute ethanol, and an aliquot was added directly to the blood or plasma and a separate aliquot was placed into a counting vial.

RESULTS

In developing the method for the measurement of tocopherol in erythrocytes, the most difficult problem to

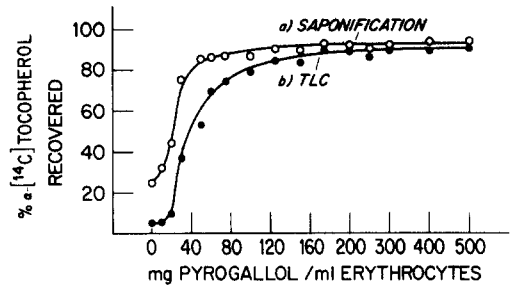

Fig. 1. Percentage recovery of added α-[^{14}C]tocopherol from human erythrocytes as a function of added pyrogallol *a*) after saponification and *b*) after saponification and TLC.

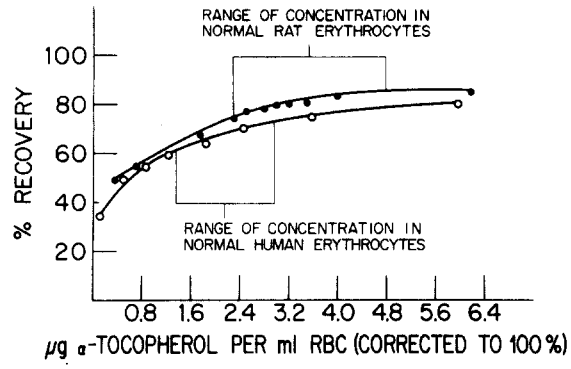

Fig. 2. Percentage recovery of [^{14}C]tocopherol added to tocopherol-free human and rat erythrocytes.

overcome was the extensive loss of tocopherol that occurred during the saponification and extraction steps. This problem was finally solved by adding unusually large amounts of an antioxidant to protect tocopherol from oxidation. Fig. 1 shows the recovery of α-[^{14}C]tocopherol from red blood cells after saponification and TLC when different amounts of pyrogallol are present. It should be noted that 10 μg of nonradioactive carrier tocopherol was added in each determination. The results indicate that about 180 mg of pyrogallol/ml of RBC is needed for the protection of erythrocyte tocopherol during saponification. It was also observed that L-ascorbic acid at about half the concentration of pyrogallol is equally protective.

This method actually measures the total tocopherol present since the TLC solvent system does not separate α-tocopherol from β-, γ- or δ-tocopherol. The TLC step is included so that the tocopherols are separated from other hexane-soluble material that might interfere with the colorimetric reactions.

The recovery of tocopherol after TLC is not quantitative when the amount of tocopherol being measured is small; this is a particular problem in experiments on red blood cell tocopherol. To demonstrate that recovery is a function of the amount of tocopherol, experiments were carried out using red blood cells from severely vitamin E-deficient rats and red cells from a patient with abetalipoproteinemia. Measurable amounts of tocopherol were not present in either sample of red cells. Different amounts of α-[^{14}C]tocopherol were added to the red cells and the analysis was carried out to completion. Fig. 2 shows the results, measured by ^{14}C recovery. The data indicate that the recovery of the added tocopherol to the RBC from the patient with abetalipoproteinemia was somewhat less than the recovery of the added tocopherol to the RBC from severely vitamin-E deficient rats. The range of concentration of tocopherol in normal human and in normal rat erythrocytes is shown on each of the two curves. The percentage of recovery for normal RBC based on those experiments with tocopherol-deficient RBC is from 60 to 70% for human RBC and 72 to 85% for normal rat RBC.

TABLE 1. Tocopherol values in plasma and in RBC and the percentage recovery measured by analysis of isotopically labeled tocopherol

Date	Plasma			RBC		
	Uncorrected	Recovery	Corrected	Uncorrected	Recovery	Corrected
	μg/ml	%	*μg/ml*	*μg/ml RBC*	%	*μg/ml RBC*
A[a]	15.57	82	17.45	2.76	85	3.24
	16.12	90	17.89	2.77	80	3.48
	16.01	91	17.59	2.29	79	2.89
	16.11	97	16.61	2.54	79	3.20
	16.14	93	17.23	2.63	74	3.54
	16.07	91	17.62	2.58	76	3.38
Average ± SEM	16.00 ± 0.22	92	17.40 ± 0.45	2.60 ± 0.18	79	3.29 ± 0.24
B[b]						
1/72	18.7	98	19.2	2.24	64	3.50
7/10/72	15.9	86	18.5			
7/24/72	14.7	86	17.1	2.52	73	3.45
8/72	16.1	86	18.7	2.14	68	3.14
1/8/73	14.9	90	16.6	2.21	59	3.75
1/16/73	14.1	89	15.8	2.88	88	3.27
Average	15.7	89	17.7	2.40	70	3.42

[a]Analyses of six aliquots of plasma and of RBC from one subject (H. K.) to demonstrate the precision of the method.

[b]Results of six analyses of plasma and of RBC in the same subject (H. K.) over a 12-month period. All samples were run in duplicate and results agreed within 5% for plasma and 10% for RBC.

Table 1 (A) presents data on the reproducibility of the method in 6 aliquots of plasma and RBC from one subject. The directly determined plasma and RBC values are shown along with the percentage recovery based on the [^{14}C]tocopherol and the corrected final recovery. Plasma values directly determined averaged 16.0 ± 0.22 μg/ml (SEM) and corrected plasma values were 17.40 ± 0.45 μg/ml. The percentage recovery for plasma was 82–97 with an average of 92, and for RBC, 74–85, average 79. Corresponding RBC values were 2.60 ± 0.18 μg/ml and 3.29 ± 0.24 μg/ml. Section B gives the results of six analyses in one subject (H.K.) over a 12-month period. The recovery of tocopherol in plasma averaged 89% and in RBC 70%.

Table 2 presents the plasma and RBC tocopherol levels of seven normal human subjects, six subjects with hyperlipoproteinemia, and one subject who was taking large supplementary doses of the vitamin. In section A the values for the plasma and RBC determined directly are shown as well as the percentage recovery of added [^{14}C]tocopherol. The range in values for the plasma and RBC tocopherol levels were 9.5–15.7 μg/ml and 1.41–3.25 μg/ml of RBC respectively, for normals, and 16–28 μg/ml and 1.54–4.24 μg/ml of RBC for hyperlipoproteinemic subjects. These latter subjects tended to have higher tocopherol levels in both plasma and RBC than were found in normal subjects. The recovery of [^{14}C]tocopherol in plasma averaged 86%, and in RBC 74%, for the normal subjects. The ratio of RBC to plasma tocopherol in normals was 0.12–0.29 with an average of 0.18. It is apparent that although the hyperlipoproteinemic subjects had almost double the amount of tocopherol in plasma and in RBC, their RBC/plasma ratios were quite similar to that for normals, 0.16 vs. 0.18. The same observation is evident in the subject M.K., who ingested large quantities of vitamin E and whose tocopherol levels were nearly four times those of normals. Section B gives the plasma and RBC tocopherol levels corrected to 100% recovery. These values show a slightly higher average for plasma and RBC tocopherol levels and also a slightly higher RBC/plasma tocopherol ratio.

In experiments where human subjects were fed large oral doses of vitamin E (2 g with 90 ml of heavy cream) it was observed that the ratio of RBC to plasma tocopherol was relatively constant throughout a 48-hr period. Fig. 3 shows the results in one normal human subject.

Patients with the rare genetic disease abetalipoproteinemia have such very low levels of vitamin E that when using the standard procedure for the spectrophotometric assay, that is, 1 ml of plasma and 2 ml of washed RBC, the tocopherol levels were barely detectable. However, by combining four hexane extracts, it was possible to measure the vitamin E levels in four of these patients (see Table 3). The RBC tocopherol levels ranged from 0.13 to 1.02 μg/ml, the plasma levels from 0.11 to 0.82 μg/ml, and the RBC-to-plasma ratios from 0.39 to 2.0.

The relative distribution of vitamin E between RBC and plasma was determined by incubating whole blood at 37°C for 4 hr in a silanized flask containing radioactive tocopherol dispersed on its surface. To ensure complete

TABLE 2. Tocopherol values in plasma and in RBC of normal human and hyperlipoproteinemic subjects

Subject	A[a]					B[b]		
	Plasma[c]		RBC[c]		Ratio RBC/Plasma			
	Uncorrected	Recovery	Uncorrected	Recovery	Uncorrected	Plasma Corrected	RBC Corrected	Ratio RBC/Plasma Corrected
	μg/ml	%	μg/ml RBC	%		μg/ml	μg/ml RBC	
H. K.	15.7	89	2.40	70	0.15	17.7	3.42	0.19
L. B.	11.2	79	1.41	66	0.12	14.2	2.14	0.15
E. M.	9.5	76	1.51	71	0.16	12.5	2.13	0.17
D. S.	13.8		1.82		0.13			
F. B.	14.2		2.24		0.16			
D. K.	11.7	92	2.60	85	0.22	12.7	3.05	0.24
L. H.	11.1	94	3.25	86	0.29	11.8	3.80	0.32
Average ± SEM	12.5	86	2.18	74	0.18 ± 0.06	13.8	2.91	
Hyperlipoproteinemic Subjects								
G. M.	19.1	92	2.45	71	0.13	20.8	3.45	0.17
J. S.	16.3		1.54		0.10			
H. S.	28.2		3.54		0.13			
N. K.	21.1		3.89		0.19			
T. S.	28.4	90	4.12	78	0.15	31.5	5.29	0.17
M. M.	16.5	85	4.24	87	0.26	19.5	4.90	0.25
Average ± SEM	21.6	89	3.30	79	0.16 ± 0.06	24.0	4.58	
M. K.[d]	60.5	79	6.16	63	0.10	71.3	9.79	0.14

[a] The plasma and RBC vitamin E levels (μg/ml) and ratio of RBC/Plasma in a group of normal human subjects, in six subjects with hyperlipoproteinemia, and in one subject ingesting 3 g of vitamin E daily.

[b] The data in section A calculated to 100% recovery based on the recovery of added tracer amounts of α-[^{14}C]tocopherol.

[c] All samples were run in duplicate and results agreed within 5% for plasma and 10% for RBC.

[d] This subject was ingesting 3 g of supplemental vitamin E daily.

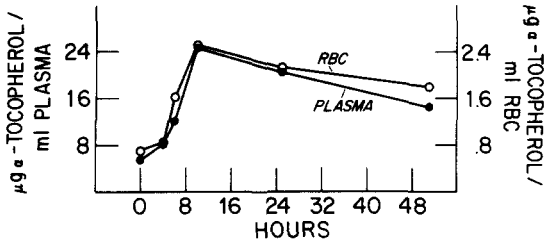

Fig. 3. Plasma and erythrocyte levels of α-tocopherol (μg/ml) after administration of 2 g of α-tocopheryl acetate in 90 ml of heavy cream to a normal human subject. The RBC ordinate is on the right and the plasma ordinate is on the left.

equilibrium, the blood was then transferred to another silanized flask for an additional 2–4 hr of incubation. Table 4 shows the results of this study and compares the ratios of RBC to plasma tocopherol in a normal subject and in a subject on a high vitamin E intake. The ratio of radioactive tocopherol in the RBC to the plasma (in dpm/ml) for the normal subject is 0.14; this value compares well with the ratio obtained by spectrophotometry in the same subject, 0.17. A similar study carried out on blood from a human subject who had been ingesting large amounts of vitamin E daily gave an RBC-to-plasma ratio of 0.16 by radioactive measurement and 0.14 by the spectrophotometric method.

The studies with rats were carried out with groups of at least five animals, and blood from each group was pooled. The RBC-to-plasma tocopherol ratio was 0.39 by the spectrophotometric method where the RBC and plasma levels were 3.8 and 9.8 μg/ml, respectively. The in vitro incubation in a silanized flask of rat blood with α-[^{14}C]tocopherol gave an RBC-to-plasma ratio of 0.36. With the animals, it was possible to feed radioactive vitamin E by gavage and to harvest blood 12 hr later. The radioactivity in the RBC and plasma was directly measured and the RBC-to-plasma ratio from the feeding experiment was 0.38 (Table 4).

TABLE 3. Tocopherol levels of RBC and plasma for subjects with abetalipoproteinemia

		A[a]			B[b]		
Date	Subject	RBC	Plasma	RBC/ Plasma	RBC	Plasma	RBC/ Plasma
		μg/ml	μg/ml		μg/ml	μg/ml	
1/72	A. V.	0.4	0.82	0.49			
4/72		0.16	0.41	0.39			
6/72		0.24	0.11	2.18	0.32	0.20	1.6
8/72[c]		1.02	0.7	1.46	1.36	0.96	1.42
2/72	M. S.	0.22	0.27	0.82			
2/72	D. P.	0.20	0.24	0.83			
2/72	G. F.	0.13	0.21	0.62			

[a] Values measured directly from spectrophotometric method.
[b] Values corrected to 100% tocopherol recovery by addition of [^{14}C]-tocopherol.
[c] Dose of supplemental oral tocopherol was doubled for 2 wk prior to this measurement.

DISCUSSION

Several different techniques have been used for the measurement of tocopherol in plasma. These have included spectrophotometric (13, 14), spectrofluorometric (15), and gas–liquid chromatographic assays (12) of the isolated and extracted tocopherol after a number of steps usually involving paper or thin-layer chromatography. Only a few observations on the content of tocopherol in red blood cells were published before 1970. Kaludin (10) measured α-tocopherol in the erythrocytes of rats by paper chromatography and a colorimetric measurement of the isolated tocopherol. Sternberg and Pascoe-Dawson (7) and Krishnamurthy and Bieri (8) published plasma:erythrocyte ratios of radioactivity after feeding radioactive α-tocopherol. An elaborate and time-consuming method for determining α-tocopherol in erythrocytes by gas–liquid chromatography was published by Bieri et al. in 1970 (12). Their method required the introduction of a known amount of radioactive tocopherol to blood with each determination, an extraction procedure of the washed erythroctyes, the

TABLE 4. Comparison of the RBC-to-plasma ratio of tocopherol by direct measurement, after incubation of whole blood with radioactive tocopherol, and after feeding radioactive tocopherol

	A[a]			B[b]			C[c]		
	Tocopherol Content in:		Ratio of Tocopherol in RBC/ Plasma	Distribution of Radioactive Tocopherol after Whole Blood Incubations		Ratio of Radio-activity in RBC/ Plasma	Distribution of Radioactivity in Whole Blood after Ingesting Radioactive Vitamin E		Ratio of Radio-activity in RBC/ Plasma
	RBC	Plasma		RBC	Plasma		RBC	Plasma	
	μg/ml	μg/ml		dpm/ml	dpm/ml		dpm/ml	dpm/ml	
Normal human subject	3.14	18.7	0.17	2.06×10^4	14.07×10^4	0.14			
Human subject with very high tocopherol levels	9.8	71.3	0.14	8.8×10^3	54.5×10^3	0.16			
Rats[d]	3.8	9.8	0.39	3.56×10^5	9.83×10^5	0.36	2.12×10^5	5.52×10^5	0.38

[a] Tocopherol levels (μg/ml) in humans and rats corrected to 100% recovery.
[b] Distribution of radioactive tocopherol in RBC and plasma after in vitro incubations of whole blood.
[c] Distribution of radioactive tocopherol after ingestion of α-[^{14}C]tocopherol.
[d] At least five animals were studied in each group and samples were pooled before analysis.

oxidation of α-tocopherol to α-tocopherylquinone, purification and separation of the quinone by TLC, and then gas–liquid chromatography of the isolated α-tocopherylquinone. Quantitation of the tocopherylquinone was carried out by comparison with an external standard, and the final result was corrected by the recovery of the initially added radioactivity. In their hands a full day was required for the analysis of two samples, or eight samples could be analyzed in 2 days (12).

At the same time as the method of Bieri et al. (12) was published, we were developing an accurate, simple, and reasonably rapid method that could be used by laboratories where gas–liquid chromatography and radioactive counting equipment were not available. It was apparent from our initial observation that when radioactive tocopherol was added to whole blood or to red blood cells, there was pronounced destruction or alteration of the tocopherol during the extraction procedure with or without a saponification step. The progressive improvement in the recovery of tocopherol, which paralleled the addition of increasing amounts of an antioxidant, resulted in a final requirement of 100 mg of pyrogallol for each milliliter of red cells of approximately 50% hematocrit. This represents an amount of pyrogallol approximately 10^5 times the tocopherol content of the erythrocyte, on a weight basis. (For ascorbic acid the value is 0.5×10^5 times the concentration of tocopherol.)

Recovery of added radioactivity after saponification and extraction of normal human erythrocytes was always greater than 90%; the recovery after TLC, elution, and colorimetric determination decreased the recovery to the range of 65–75%. From a number of experiments it appeared that the most important factor determining the percentage recovery was the total amount of tocopherol present in the erythrocytes (or in the plasma) at the start of the analysis. It therefore was appropriate to construct a graph (Fig. 2) plotting percentage recovery against the amount of tocopherol added to erythrocytes entirely deficient in tocopherol. This curve, obtained by measuring the recovery of radioactive tocopherol over a wide range of tocopherol concentrations, can be used to correct the observed tocopherol values for human or rat erythrocytes. Recoveries of tracer radioactive tocopherol added to plasma samples (of both normal humans and rats) were uniformly greater than 85%. These values have been used to calculate the RBC-to-plasma ratios, which by direct analysis gave a value of 0.18 ± 0.06 (SEM); when corrected to 100% by radioactive recovery measurements in both plasma and RBC, the ratio was 0.21 ± 0.07. These values are slightly different from those reported by Bieri et al. (12). They reported that the RBC-to-plasma ratio in 11 normal subjects was 0.244 ± 0.02, with a wide range in values for both plasma and RBC tocopherol values. A review of their data indicates that the red blood cell levels in their subjects and ours are similar, but that the plasma levels in their subjects are lower. The resulting RBC-to-plasma ratios are therefore lower. Their method measures solely α-tocopherol, while the present method measures total tocopherol. A comparison of the results in normal rats show the following pattern: our value for erythrocyte tocopherol is somewhat higher than in their animals, and the plasma values for our animals are distinctly higher than theirs; the ratio of RBC to plasma tocopherol for our animals is lower, 0.39 vs. 0.48.

The explanation for the different values in the animals may be a matter as simple as differences in the strains of rats studied or the use of different diets. We have attempted to verify our analytical results by other techniques than solely the recovery of added labeled tocopherol. In one series of studies, we placed labeled tocopherol on the walls of silanized glass flasks, added whole blood, and allowed the vitamin to pass into the plasma and red cells during a 3-hr incubation. To ensure complete equilibration, the blood was then transferred to another silanized vessel free from radioactive tocopherol and incubated for an additional 2 hr. The comparison of ratios between the direct analysis and the incubated radioactive method gave good agreement; for normal subjects, the values were 0.17 vs. 0.14; for a subject on a very high vitamin E intake, the values were 0.14 vs. 0.16; and for normal rats the values were 0.39 vs. 0.36. An additional analytical technique was possible in the experimental animals. To verify the erythrocyte-to-plasma ratio, radioactive tocopherol in oil was fed to rats, and the blood was harvested the next day. The radioactivity measured in the erythrocytes and plasma gave a ratio of 0.38.

The differences in the values of tocopherol for human subjects reported by Bieri et al. (12) and our own findings may be due to any of the following possibilities. The two studies have examined only very small groups of normal human subjects, and the values in the two groups may be truly different. We have made one attempt to compare the results of tocopherol measurement by the two methods on the same erythrocytes. In collaboration with Dr. Barbara Underwood of the Institute of Nutrition, Columbia University, New York, five samples were studied. The spectrophotometric and gas–liquid chromatographic methods (values corrected to 100% recovery) gave average values of 1.74 and 1.91 μg/ml, respectively, for four samples; these results are in close agreement. The mean variance for these four samples was only 0.17 μg/ml, and for all five samples the mean variance was 0.55 μg/ml. An extended comparison of the two methods on the same samples might minimize the reported variation in different populations.

The erythrocyte-to-plasma ratio of tocopherol appears to fall within a relatively narrow range despite a wide range of plasma or erythrocyte concentration. This is ap-

parent in the subject with a plasma value six times that of the normal subject due to an intake of 3 g of tocopherol/day. However, in the four subjects with abetalipoproteinemia (Table 3), the red cells contained a far higher amount of tocopherol in proportion to plasma tocopherol than was seen in any normal human subject. The results in these four subjects are similar to the values reported by Bieri and Poukka (16) in four other patients with abetalipoproteinemia. That the acanthocytes can take up additional tocopherol was demonstrated in experiments in which the erythrocytes of these patients were incubated in plasma of subjects with high plasma and erythrocyte tocopherol levels due to high intake of vitamin E (17). The tocopherol level in these acanthocytes rose to almost the same value as in the erythrocytes in the blood of the donor subject.

The spectrophotometric method has proved to be rapid, convenient, and reproducible. We routinely analyze as many as 20 blood samples in a single day. This method should be of interest to laboratories in which nutrition and vitamin level studies are undertaken.

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