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Improved Method for Microdetermination of Plasma Vitamin E in Laboratory Rats

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Abstract □ The method generally used to assay human serum for vitamin E (tocopherol) was found to be inapplicable to rat plasma. However, a modified fluorometric method was suitable for both human and rat plasma. This microassay is relatively rapid and reproducible and was utilized to monitor the onset of the tocopherol-deficiency state in rats fed a vitamin-free diet.

Keyphrases □ Vitamin E—improved microdetermination, rat plasma □ Tocopherol—improved microdetermination, rat plasma □ Fluorometry—microdetermination of vitamin E, rat plasma

Problems associated with the analysis of vitamin E (tocopherol) in biological fluids obtained from laboratory animals include the small sample size and the endogenous materials that interfere with tocopherol determinations. The present study describes a modified procedure that improves the fluorometric analysis (1) of tocopherol in humans. The modified microassay is relatively rapid and extremely reproducible, and it may be used for small laboratory animals as well as humans.

EXPERIMENTAL

Rats, Sprague-Dawley[1], weighing 156 ± 10 g, were divided into three groups of 60 each. The normal control animals were fed ordinary laboratory chow[2], while the others were fed either a tocopherol-deficient diet (deficient group) or the deficient diet supplemented with 20 μg/g of α-tocopheryl acetate (supplemented control group). Food and water were allowed *ad libitum*. The composition of the deficient diet was as suggested by Bieri and Privel (2). Both the deficient and supplemented diets[3] were prepared in powdered form in these laboratories. The rat food was assayed colorimetrically at 534 nm for tocopherol content by the method of Bieri (3).

To establish whether the decline in tocopherol plasma levels was a result of the diet and not to a lack of food intake, the rate of animal growth as well as food consumption was monitored.

The method of analysis for free and total tocopherol plasma levels in rats was a modification of the fluorometric procedure of Hansen and Warwick (1) which was suggested for humans. These authors used a 0.1-ml serum sample to which were added 1 ml of water, 1 ml of ethanol, and 2 ml of hexane. The mixture was shaken with a mechanical shaker and centrifuged; then a portion of the clear supernatant layer was assayed fluorometrically, against a reagent blank, for free tocopherol at 295 and 340 nm, the wavelengths of maximum excitation and emission, respectively. Total tocopherol (free plus acetate) was determined by redissolving 1 ml of the remaining supernate, after evaporation to dryness, in 2 ml of hexane and adding 0.5 ml of a solution containing 15.0 mg $LiAlH_4$ dissolved in 10 ml of anhydrous ether. Three milliliters of 0.1 N H_2SO_4 was added, the solution was mixed and centrifuged, and the fluorescence was measured.

In this study the assay procedure was modified by increasing the sample size to 0.2 ml of plasma and reducing the volume of

[1] Blue Spruce Farms, Altamont, New York.

[2] Ralston Purina Co., St. Louis, Mo.

[3] All dietary constituents were purchased from General Biochemicals, Chagrin Falls, Ohio, with the exception of the stripped lard which was obtained from Eastman Kodak Co., Rochester, N.Y. All materials were used as received.

Table I—Free Tocopherol Content of Human Plasma[a]

Subject	Mean Plasma Level[b] ± *SD*	
	Method of Hansen and Warwick (1)	Modified Method
1[c]	23.0 ± 0.51	25.8 ± 0.63
2	13.0 ± 0.54	13.8 ± 0.31

[a] No dependence on agitation intensity could be detected using human plasma. [b] Mean of six determinations for each subject. No statistical difference (Student's *t* test) in tocopherol levels exists between the two methods of analysis. [c] Subject 1 supplements a normal diet with 100 mg/day of tocopherol.

water from 1.0 to 0.2 ml. It was found that 0.5 ml of a solution of 200–300 mg of finely powdered lithium aluminum hydride dissolved in 20 ml of cold freshly distilled ether was necessary for complete reduction of plasma tocopherol. A reciprocating shaker[4] and a high agitation mixer[5] of the type recommended by Hansen and Warwick (1) were used.

All fluorometric measurements were made with a spectrophosphormeter[6] at 295 and 330 nm, the wavelengths of maximum excitation and emission, respectively.

Tocopherol recovery determinations were carried out using the modified procedure by adding a quantity of the vitamin to rat plasma. The amount of tocopherol added to blank plasma samples ranged from 0.5 to 10 μg/ml and represented quantities of tocopherol in the plasma of rats and humans found during these experiments. A mean amount ± *SD*, representing 97.6 ± 5.3% of the vitamin added to the plasma, was recovered after accounting for endogenous tocopherol.

Plasma samples were usually assayed immediately; however, storage of the samples in the frozen state for as long as 3 weeks did not result in apparent tocopherol loss. All reagents were purified according to the method of Hansen and Warwick (1).

RESULTS AND DISCUSSION

Analysis of the normal laboratory chow and the supplemented diet yielded tocopherol values of 17.0 and 17.5 μg/g food, respectively. Analysis of the deficient diet yielded tocopherol levels below the limits of the assay procedure (0.33 μg/g diet). Food consumption for the control diet (supplemented group) and the test diet (deficient group) was found to be statistically[7] identical, *i.e.*, 17.3 ± 0.4 and 16.9 ± 0.5 g/rat/day, respectively, and within the range for this strain of rat fed ordinary laboratory chow. The gain in body weight (Fig. 1) was identical for the supplemented control and deficient animals; however, both groups showed a significant weight gain difference from the normal control animals. This difference in growth patterns for animals fed the diet of Bieri and Privel (2) has not been previously reported and illustrates that one should be cautious in the choice of a control diet when performing vitamin-deficiency studies. The results indicate that both the supplemented and deficient diets were equally well accepted by the animals. Visual observation of each experimental group over 8 months did not reveal any gross differences among the animals.

The results of the analysis of human plasma by either the method of Hansen and Warwick (1) or the modified method were found to be extremely reproducible and within the normal range reported by these authors (Table I).

Similar results were not obtained when rat plasma was analyzed for free tocopherol content. Table II shows that the method of Hansen and Warwick yielded low plasma values for free tocopherol when a reciprocating shaker was used and abnormally high values when a high agitation mixer was used. The modified method, however, resulted in reproducible values regardless of the shaking procedure. The values obtained with the modified method were within the normal range of values reported previously by Bieri (4) using an independent method of analysis.

With rat plasma the modified extraction procedure resulted in a heavy, flocculent precipitate, which is believed to consist mainly of plasma proteins; only a slight haziness occurred when extraction was accomplished by use of the Hansen and Warwick (1) technique. It appears, therefore, that the modified assay technique is relatively insensitive to agitation intensity because plasma proteins were efficiently precipitated and the vitamin was released from its binding sites. In the Hansen and Warwick procedure, protein precipitation did not appear to be complete. This resulted in either incomplete extraction when weak agitation was used for partitioning or extraction of highly fluorescent substances together with the free tocopherol when a high agitation mixer was used in the assay. Table II shows that an apparently low tocopherol content (1.7 μg/ml) was observed with the Hansen and Warwick procedure using the reciprocating shaker and an abnormally high value (19.5 μg/ml) was found when the high agitation mixer was employed. Abnormally high values were also obtained using blank plasma samples. Tryptophan is the only component nor-

Table II—Free Tocopherol Content of Normal Rat Plasma[a]

	Method of Hansen and Warwick (1)	Modified Method
Reciprocating shaker[b]	1.70 ± 0.28[c] (4)	4.65 ± 1.06 (4)
High agitation mixer[d]	19.52 ± 5.71[c] (6)	4.76 ± 1.3 (11)

[a] Micrograms per milliliter ± *SD*. Values in parentheses denote number of determinations. [b] Extraction time was found to be maximal after 20 min at the fastest setting. [c] Statistically different from all other values (Student's *t* test); $p < 0.05$. [d] Extraction time was found to be maximal after 3 min.

Table III—Total Tocopherol Content of Normal Rat Plasma[a]

	Method of Hansen and Warwick (1)	Modified Method
Reciprocating shaker[b]	2.29 ± 1.10[c] (7)	4.60 ± 0.11 (4)
High agitation mixer[d]	4.85 ± 0.78 (4)	5.05 ± 0.84 (4)

[a] Micrograms per milliliter ± *SD*. Values in parentheses denote number of determinations. [b] Extraction time was found to be maximal after 20 min at the fastest setting. [c] Statistically different from all other values (Student's *t* test); $p < 0.005$. [d] Extraction time was found to be maximal after 3 min.

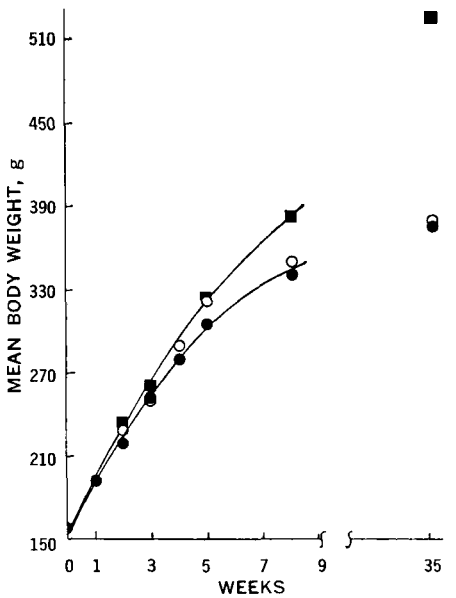

Figure 1—*Growth patterns of rats maintained on test diets. Key:* ●, *deficient;* ○, *supplemented; and* ■, *regular chow.*

[4] Variable-speed reciprocating shaker, Eberbach Corp.
[5] Vortex-Genie, Scientific Industries.
[6] Aminco-Keirs, with fluorescent attachment.
[7] Student's *t* test, $p > 0.05$.

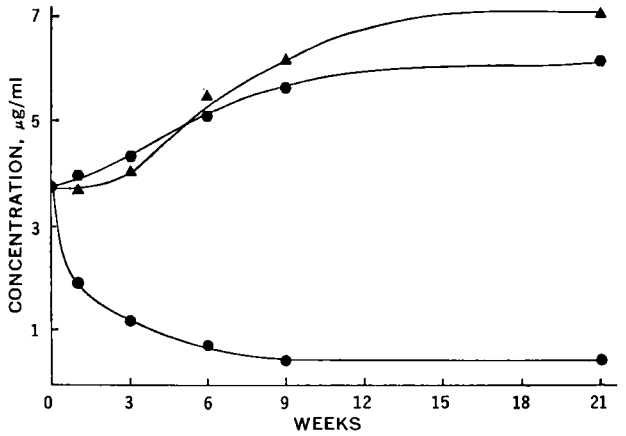

Figure 2—*Tocopherol plasma levels of rats maintained on test diets. Key: ●, deficient group; ▲, supplemented control; and ●, normal control.*

mally present in plasma extracts in concentrations high enough to show a measurable fluorescence (5). A twofold greater tryptophan concentration exists in rat blood as compared to human blood (6), and it is not unreasonable to assume that the high fluorescent values were caused by tryptophan. These abnormally high fluorescent values did not occur in the analysis of total tocopherol, probably because the fluorescent materials were not transferred to the organic phase after treatment with lithium aluminum hydride (Table III). This phenomenon also does not occur in the analysis of free tocopherol by use of the modified method where protein precipitation is more efficient nor with human plasma, probably due to differences in plasma constituents (6, 7). When using human plasma, both methods yielded the same degree of apparent protein precipitation.

Table III illustrates that the method of Hansen and Warwick resulted in incomplete extraction of tocopherol from rat plasma when a reciprocating shaker was used. The modified method, however, gave reproducible results regardless of agitation intensity.

The values for free and total plasma tocopherol in rats appear to be identical when determined by the modified method. Although it was not possible in this study to compare the ratio of free to total tocopherol using the Hansen and Warwick procedure, these authors could not demonstrate a statistical difference between concentrations of the two forms (free and acetate) of the vitamin in human serum. Since the concentration of free and total tocopherol in serum or plasma appears to be identical, one can accurately follow the time course of the vitamin in the body, using the modified method, by measuring only the free form and thus eliminating the longer procedure of the reduction of tocopheryl acetate.

The data shown in Table IV further demonstrate the utility of the modified assay procedure for the analysis of rat plasma tocopherol levels. Tocopherol-deficient rats can be differentiated from normal animals by use of this assay method; moreover, tocopherol depletion in the deficient group was similar to that observed by Bieri (4). Analysis of the same data, for the first 6 weeks, by the method of Hansen and Warwick resulted in an apparent tocopherol plasma content of approximately 2.5 μg/ml regardless of length of time on diet or deficiency state.

The results shown in Fig. 2 suggest that, with a reasonably deficient diet, one can expect that rats will deplete most of their body tocopherol stores in approximately 6 weeks. This finding is confirmed by the work of other authors using various physiological parameters such as kidney autolysis (8), muscular dystrophy (9), oxygen consumption in isolated perfused hearts (10), decrease in testicular weights (11), and composition of nonphosphatides from adipose tissue (12).

Table IV—Free Tocopherol Analysis as a Function of Time Using the Modified Method

Weeks on Diet	Normal[a] Control Group	Supplemented[a] Control Group	Deficient[a] Group
0	3.8 ± 0.7	3.8 ± 0.7	3.8 ± 0.7
1	4.1 ± 0.6[b]	3.7 ± 0.7[b]	1.9 ± 0.6
3	4.5 ± 0.5[b]	3.8 ± 0.7[b]	1.2 ± 0.3
6	5.1 ± 1.6[b]	5.5 ± 1.2[b]	0.7 ± 0.3
9	5.6 ± 0.5[b]	6.2 ± 0.8[b]	0.4 ± 0.2
21	6.2 ± 1.6[b]	7.0 ± 2.8[b]	0.4 ± 0.2

[a] Mean of five animals (μg/ml ± *SD*). [b] Statistically different from deficient groups (Student's *t* test); $p < 0.05$.

SUMMARY

It was observed that the method of Hansen and Warwick (1) for human serum tocopherol determinations could not be used without modification to assay the vitamin in rat plasma. Their procedure does not efficiently precipitate plasma proteins; it results in poor tocopherol extraction when using a low agitation shaker and in the transfer of fluorescent impurities when using a high agitation mixer. The modified procedure, by increasing the organic to aqueous volume ratio, produces efficient precipitation of plasma proteins without dependence on shaking intensity. The modified procedure was used to differentiate between normal and tocopherol-deficient rats, and the results were essentially identical to those found by other workers using an independent method of analysis (4).

No difference was observed between the Hansen and Warwick procedure and the modified method in the analysis of human plasma tocopherol levels.

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