An evaluation of vitamin E status in premature infants\textsuperscript{1-3}

Gary R Gutcher, MD, William J Raynor, PhD, and Philip M Farrell, MD, PhD

ABSTRACT Prematurely born, low birth weight infants are abnormal by their very existence ex utero. Thus, the well-documented finding in such infants of low plasma vitamin E concentrations when compared to the adult poses philosophical and pragmatic difficulties as to whether or not a true deficiency state exists: do these low levels represent age-adjusted reference values or do they in fact represent a deficiency state, warranting treatment? We examined multiple measures of vitamin E status in 62 prematurely born, low birth weight infants in order to address this issue. Mathematical and statistical modeling of these measures during the first 21 days of life lead us to conclude that the ex utero antioxidant protective role of vitamin E is best achieved at plasma concentrations of tocopherol very close to those observed in the adult; specifically, when total tocopherol is \(>0.64\) mg/dl and \(\alpha\)-tocopherol is \(>0.50\) mg/dl. Thus, at birth, a true deficiency in vitamin E exists for most preterm, low birth weight infants and early treatment is warranted. 


KEY WORDS Premature infants, vitamin E, tocopherol, high-pressure liquid chromatography

Introduction

The usual dietary intake of tocopherol in the United States is adequate to render vitamin E deficiency rare among adults and children except in instances of chronic intestinal malabsorption or in preterm low birth weight infants \(\textsuperscript{1}\). However, since the deficiency state has been associated with hematological \(\textsuperscript{2}\) and neuromuscular \(\textsuperscript{3}\) disorders, the definition of human nutritional status with regard to tocopherol has assumed some importance. Most assessments of human vitamin E status have surveyed single measures of tocopherol in groups of healthy adults or children. The various approaches used have included measures of in vitro hemolysis tests with hydrogen peroxide as an oxidant stress \(\textsuperscript{4}\), plasma concentrations of total tocopherol (TT) \(\textsuperscript{5}\), \(\textsuperscript{6}\), plasma concentrations of \(\alpha\)-tocopherol (AT) \(\textsuperscript{7}\), or the computation of plasma tocopherol/lipid ratios \(\textsuperscript{8}\). Most investigations have not assessed the relative quantities of the various tocopherol isomers, failing to recognize or possess the ability to distinguish tocopherol isomers of differing biological activity \(\textsuperscript{9}\). Because vitamin E isomers such as \(\beta\)-, \(\delta\)- and \(\gamma\)-tocopherol are now known to be present in human breast milk, commercial formulas, and other nutritional products (but only provide a fraction of the antioxidant activity available as the generally more abundant AT), it has become important to fractionate blood tocopherols by reliable new techniques such as high pressure liquid chromatography \(\textsuperscript{10}\).

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Previously published surveys of plasma tocopherol concentrations in preterm infants have uniformly detected levels far below those found in healthy adults and have coincidentally shown high rates of oxidant-induced in vitro erythrocyte hemolysis (1). It is not clear whether these low levels define an age-adjusted range of normal for the preterm infant or whether they in fact define a deficiency state. Nonetheless, attempting to discriminate as precisely as possible between tocopherol-sufficient and tocopherol-deficient low birth weight infants is an increasing imperative as a theoretical and experimental basis is being developed which advocates the use of parenteral tocopherol in pharmacological doses as a form of therapy to ameliorate or totally treat a variety of neonatal disorders. Specifically, several studies have now suggested that retrolental fibroplasia which occurs in preterm infants who have usually received therapeutic oxygen may be ameliorated, although not totally prevented, by the administration of large doses of parenteral tocopherol (11–15). Yet others have purported to describe a salutary effect of tocopherol on bronchopulmonary dysplasia (16), another disease related to oxygen administration that occurs almost exclusively in preterm infants, although several subsequent reports (17–20) were unable to confirm any therapeutic effect. If this poorly characterized tocopherol “deficiency” is to be treated, a comprehensive understanding of the tocopherol status of preterm infants must come first. Consequently, this survey in preterm, low birth weight infants was undertaken for two purposes: 1) to evaluate tocopherol isomers specifically and relative to total plasma lipids and, 2) to define the critical values of tocopherol measures for vitamin E deficiency.

Methods

Patient material and specimen collection

Infants admitted to the Special Care Nursery at Madison General Hospital during November 1981 through January 1983 were eligible for enrollment if: 1) birth weight was <2.5 kg, 2) no major congenital anomalies were present, and 3) parental consent was obtained. The study had received prior approval by the University of Wisconsin Human Research Committee and the Madison General Hospital Institutional Review Board. Sixty-two patients were enrolled, as described in Table 1. During the course of the survey the clinical and nutritional management of the patients was at the discretion of the attending physician. Therefore, the varied state of illness as well as the timing and nature of nutrition provided an array of resultant tocopherol status measures. Thus, some very ill infants received various doses of AT acetate intravenously as a multivitamin preparation (MVI-12, USV Pharmaceuticals, Inc) administered during total parenteral nutrition while other infants were well enough to receive vitamin E only as the enteral dose provided by human breast milk or various commercial formulas and yet others received supplemental oral tocopherol as AT acetate, 25 IU/day.

Blood samples were obtained via indwelling umbilical catheters when present or by antecubital venipuncture with 2 ml collected in EDTA-treated tubes on days 1, 3, 7, 10, 14, and 21 of life. In most instances, umbilical cord blood collected immediately after cord clamping was used as the “day 1” sample. Plasma was immediately separated from erythrocytes by centrifugation; the plasma was frozen for later analysis while a refrigerated (4°C) sample of erythrocytes was used in the peroxide-induced erythrocyte hemolysis assay within 12 h of collection.

Biochemical Assays

Assessment of vitamin E status was carried out using: 1) a high pressure liquid chromatography-based measurement of tocopherol isomers including AT, 5- tocopherol and 6-tocopherol fractions in plasma extracts; 2) determination of total lipids in plasma as a reference base; and 3) measurement of erythrocyte hemolysis in 2% hydrogen peroxide. Tocopherol isomers were quantitated according to a modified version of the method of Bieri et al (10) established in our laboratory using a Perkin-Elmer LC-85 instrument with a C-18 reverse-phase column and a variable wavelength detector. The method involves extraction of 0.2-ml samples of plasma by mixing with 0.2 ml ethanol (containing the internal standard of 50 μg/ml AT acetate) and 0.3 ml hexane. The extracts were agitated, the tubes were then centrifuged, and the hexane phase was removed and evaporated under nitrogen. The residue was then injected onto the C-18 column. AT was completely separated from the β + γ- and δ-tocopherol region and all other tocopherol isomers.

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each isomeric fraction was readily quantitated at 292 nm using AT acetate as the internal standard (21). On replicate analyses of normal adult plasma the coefficient of variation for the combined extraction and high pressure liquid chromatography procedure was 5.6 and 6.5% for AT and β + γ-tocopherol, respectively. A pilot survey had indicated that no AT acetate could be detected in the plasma of infants receiving supplemental AT acetate. Total tocopherol was calculated as the sum of AT and β + γ-tocopherol; δ-tocopherol was excluded from the calculation of total because it was generally not present and its biological activity is only 1% that of AT (9).

Total plasma lipids were measured by a modification (22) of the technique of de la Huerga et al (23). This method involves extracting 0.1 ml plasma with 1.9 ml ethyl ether/ethanol (1:3), evaporating half the extract after centrifugation, and measuring the turbidity of the sample in a mixture of p-dioxane/sulfuric acid. In order to use this assay on such a scale, it has been necessary to establish a new standard curve with samples of human plasma that were also analyzed for total lipid content with colorimetric and fluorimetric procedures.

Erythrocyte hemolysis in hydrogen peroxide was performed as follows: fresh, saline-washed erythrocytes were incubated without agitation as 5% suspensions in hydrogen peroxide (2% initial concentration). After 3 h incubation at 37°C, the samples were centrifuged and absorbance at 575 nm measured to determine the extent of Hb release. Results are expressed as a percentage of total Hb release detected in concurrently run erythrocyte suspensions that were incubated in distilled water. This technique was adapted from that of Horwitt et al (24) as described elsewhere (25) and yields less than 5% hemolysis with suspensions of red cells taken from healthy adults with normal plasma tocopherol concentrations. In 35 of our patients, the erythrocyte hemolysis in hydrogen peroxide was also performed in an incubation medium containing 0.01 M glucose which is known to reduce the hemolysis in conditions such as selenium deficiency where nontocopherol antioxidant systems are altered (26).

**Mathematical and statistical analysis**

We used mathematical modeling of the measures of tocopherol status in an effort to maximize our ability to define the critical values of normal. Our assessment proceeded on the basis of the following explicit assumptions: 1) each measure of plasma tocopherol concentration (total or isomeric) reflects total body tocopherol status; 2) the peroxide-induced erythrocyte hemolysis assay reflects membrane antioxidant integrity and tocopherol is the major component of this antioxidant system; 3) the likelihood of accurately discriminating tocopherol deficiency from sufficiency will be enhanced to the extent that one can maximize agreement of the various measures of tocopherol status; and, 4) the already defined critical values of tocopherol status for adults and children may not be accurate or relevant in the preterm low birth weight infant.

The data describing the relationships between percentage hemolysis, the values of TT and AT and total lipid, as well as the calculated total tocopherol/total lipid (TT/TL), ratio were analyzed using the statistical computing packages MINITAB for basic analysis and regressions and GLIM for logistic regression analyses. Since hemolysis is measured as a percentage and varies over a wide range (0 to 75%), the analyzed values were transformed to a logistic scale before analysis (27). This transformation is of the general form logarithm [%.hemolysis/(100-% hemolysis)] for percentage data and allows the use of standard regression techniques on data of this type. After the transformation, all variables still showed noticeable heteroscedasticity. However, the effect of this was believed to be minor and standard least squares techniques were used to fit the models reported below. Transformations of the independent variables were selected to linearize the relationship between percentage hemolysis and the analyzed variable.

A logistic regression analysis of the probability of an infant having an abnormal value of percentage hemolysis was also performed. Here, abnormal was defined using the adult critical value of >5%. The first regression analysis focuses on predicting the exact value of percentage hemolysis, while subsequent analyses predict abnormality without regard to the actual level. As before, transformations of the independent variable were chosen during an exploratory analysis.

A final analysis studied the relationships between the values of percentage hemolysis as originally measured and percentage hemolysis in the presence of 0.01 M glucose. As before, the observed values were transformed using a logistic transformation before analysis.

Further statistical analyses involved the assessment of critical values to correctly classify an infant; the overall ability of a test to correctly classify a person as normal or abnormal is generally referred to as the total agreement or efficiency of the test (28). The efficiency is measured as

\[
\text{efficiency} = \frac{\text{true positives} + \text{true negatives}}{\text{total number of observations}} \times 100.
\]

This can quite accurately describe the overall effect of changing the criteria for distinguishing normal from abnormal values for a fixed group of normals and abnormals. However, this value is dependent on the prevalence of the characteristic under study; and if the prevalence changes from group to group or if the definition of "normality" is itself under study, this measure can be artificially high or low. Further, even a totally random classification procedure, such as flipping a coin, will have an efficiency above zero. Consequently, we have used an adjusted measure of the efficiency, known as the statistic (29) to measure the agreement in our procedures. This statistic compares the observed efficiency to the efficiency that would be predicted from a totally random test with the same prevalence as the observed values. The adjusted efficiency obtained with the statistic is thus a measure of the efficiency of a test adjusted for the chance agreement that can occur.
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in any test. It is calculated as

\[ \kappa = \frac{\text{no correctly classified} - \text{no correct due to chance}}{\text{total sample size} - \text{no correct due to chance}} \times 100, \]

where the "no correctly classified" is the sum of the true positives and true negatives and "no correct due to chance" is the sum of the expected true positives and expected true negatives. The expected true negatives is calculated as expected true negatives = (total negatives) × (total normals)/sample size. Similarly, the expected true positives is calculated as expected true positives = (total positives) × (total abnormals)/sample size. Note that the kappa statistic is always smaller than the unadjusted efficiency, since it is correcting for the omnipresent chance agreement. In three of our tables the unadjusted efficiency is denoted "% agreement" while the kappa statistic is termed "% adjusted agreement."

Results

The 62 study patients are characterized in Table 1. Of the initially enrolled patients, 40 remained in the study for the entire 21 days. Of the potential 372 data points (six samples × 62 patients), 41 were lost due to death, 19 due to early discharge from the hospital, and 13 were missed due to technical blood-drawing or specimen handling problems. The 299 available data points were used in correlative analyses of tocopherol status measures. For time-trend analyses, only data obtained from patients who remained in the study for the full 21 days were used (40 patients with 225 available data points). Analyses comparing hemolysis with and without added glucose are based on a subgroup of 155 samples which were measured using both techniques.

The distribution of values in all patients for peroxide-induced hemolysis (%H), TT, AT, and the TT/TL are depicted in Table 2 for samples obtained on day 1. As earlier studies have shown (1), the mean plasma concentrations of total-tocopherol and AT are far below the lower limit of normal for adults with only a rare infant classifiable as "normal" by these criteria. It is notable that \( \beta + \gamma \)-tocopherol was detectable in the day 1 plasma samples of 41 (66.1%) of the infants. Indeed, when present, this isomeric fraction accounted for an average of 13.6% of total tocopherol with a range of 1.3 to 17.6%. We know of no normal value for these isomers as the plasma concentration in adults and older infants appears to reflect dietary tocopherol isomer intake (30-33). In 29 pairs of maternal and cord blood samples, no correlation (p > 0.3) was evident between maternal and cord blood levels for \( \beta + \gamma \)-tocopherol while for AT, \( r = 0.675 \) (p < 0.01) and for TT, \( r = 0.662 \) (p < 0.01).

Table 3 examines the time-trend in tocopherol measures for those infants who remained in the study for the full 21 days (n = 40). These data graphically display the rising plasma tocopherol levels in the population coincident with falling %H, although it appears that a significant number of infants have persistently abnormal tocopherol status during the 1st wk of life. Furthermore, for all patients on day 1, the TL concentration of 201 ± 70 mg/dl (mean ± SE) is extremely low compared to the adult and older child (22) but increases dramatically during the first 21 days. Indeed, on day 1, the TL appears to be disproportionately depressed compared to TT and AT resulting in very high TT/TL. Thus, TT/TL was above 0.6 (22) in 95% and above 0.8 (8) in 87% of infants on day 1 although %H was abnormal in 77% of these same infants.

Table 1 depicts the relationship between

TABLE 2
The tocopherol measures obtained on day 1 of life from surveyed population

<table>
<thead>
<tr>
<th>Tocopherol measure*</th>
<th>Mean</th>
<th>SD</th>
<th>Median</th>
<th>Range</th>
<th>No &quot;normal&quot; (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>0.271</td>
<td>0.114</td>
<td>0.233</td>
<td>0.132-0.646</td>
<td>3 (5)</td>
</tr>
<tr>
<td>AT</td>
<td>0.248</td>
<td>0.109</td>
<td>0.215</td>
<td>0.113-0.646</td>
<td>1 (2)</td>
</tr>
<tr>
<td>( \beta + \gamma )</td>
<td>0.024</td>
<td>0.023</td>
<td>0.026</td>
<td>0.000-0.105</td>
<td></td>
</tr>
<tr>
<td>TT/TL ratio</td>
<td>1.42</td>
<td>0.59</td>
<td>1.38</td>
<td>0.49-3.75</td>
<td>54 (90)</td>
</tr>
<tr>
<td>%H</td>
<td>24.7</td>
<td>20.6</td>
<td>18.9</td>
<td>0-71.6</td>
<td>13 (21)</td>
</tr>
</tbody>
</table>

* In this and all subsequent tables, tocopherol isomers and total lipids are expressed as mg/dl. The TT/TL ratio is as \( \mu g/dl \) tocopherol: mg/dl total lipids.
† "Normal" = TT > 0.5 mg/dl; AT > 0.5 mg/dl; TT/TL ratio > 0.8; %H < 5%. 

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TABLE 3
The time-trend analysis of tocopherol measures among infants who remained in trial for 21 days

<table>
<thead>
<tr>
<th>Day</th>
<th>n</th>
<th>% H*</th>
<th>Tocopherol†</th>
<th>Lipid‡</th>
<th>TT/TL† ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.00 M</td>
<td>0.01 M</td>
<td>TT</td>
</tr>
<tr>
<td>1</td>
<td>37</td>
<td>16.6</td>
<td>6.7</td>
<td>0.265</td>
<td>0.239</td>
</tr>
<tr>
<td>3</td>
<td>37</td>
<td>9.6</td>
<td>3.6</td>
<td>0.397</td>
<td>0.366</td>
</tr>
<tr>
<td>7</td>
<td>37</td>
<td>1.6</td>
<td>1.4</td>
<td>1.059</td>
<td>1.002</td>
</tr>
<tr>
<td>10</td>
<td>37</td>
<td>1.6</td>
<td>1.1</td>
<td>1.207</td>
<td>1.096</td>
</tr>
<tr>
<td>14</td>
<td>38</td>
<td>1.5</td>
<td>1.1</td>
<td>1.389</td>
<td>1.261</td>
</tr>
<tr>
<td>21</td>
<td>38</td>
<td>1.1</td>
<td>1.1</td>
<td>1.356</td>
<td>1.193</td>
</tr>
</tbody>
</table>

* Mean calculated on logit scale and transformed for display.
† Geometric mean.
‡ Incubated with 0.00 M and 0.01 M glucose; n = 25, 26, 25, 25, 25, and 24 for the added glucose system.

TT and TL for all data points. For data collected on day 1 only, there is good correlation between untransformed values (r = 0.46), and data transformation does not significantly improve the description. However, for data obtained from all days, the relationship depicted in Figure 1 is not linear and is best described by a log-log transformation (r = 0.51, Fig 2) rather than untransformed (r = 0.37, Fig 1). The improved correlation between transformed TT and TL represents a new finding in humans since several other investigators (34–36) have demonstrated as good or better correlation with untransformed data alone. We note, however, that the patients described by others were generally not on supplemental tocopherol and did not attain the high plasma concentrations noted in some of our infants. Indeed, it appears that it is the high TT without a correspondingly high TL that imposes the nonlinearity on the untransformed data. Surveys of healthy adults have suggested that a plasma TT of 1.6 mg/dl represented a value 2 SD above the mean (1). In Figure 1, the points with TT >1.6 mg/dl represent data from 24 patients. Of these, three had received supplemental oral AT acetate (25 IU/day) only, 16 had received 3 to 20 days of only intravenous AT acetate (1 to 5 IU/kg/day) and five had received a combination of oral and intravenous AT acetate.

The relationship between TT and %H is depicted in Figure 3, and again in Figure 4 after transformation of the data as indicated. Similar relationships (not shown) exist for AT or TT/TL versus %H. For TT and TT/TL, best fit is described by linear equations while the relationship to AT is best described.

![FIG 1. The relationship between the plasma TT and the plasma total lipids for all patients and all days. Untransformed, raw data.](https://www.sciencedirect.com/science/article/pii/S0002928909000047)
FIG 2. The relationship between the logarithm of plasma TT and the logarithm of plasma total lipids for all patients and all days.

FIG 3. The relationship between the degree of peroxide-induced hemolysis and the plasma TT for all patients and all days. Untransformed, raw data.
by a quadratic equation (Table 4). The approximate level of a tocopherol measure which would correspond to a %H of 5% was calculated to be 0.56 mg/dl for TT and 0.44 for AT. Thus it is evident that this analysis describes critical plasma tocopherol concentrations very close to the 0.5 mg/dl conventionally accepted as critical for adults and older children (1). However, the TT/TL ratio necessary to identify abnormal %H is considerably higher than the critical value of 0.8 proposed for adults (8) or 0.6 as proposed for older children (22).

An alternative approach to approximate the critical values of tocopherol deficiency uses an inference on proportions. As a first approximation, we used the conventional measures of discrimination cited in the literature for adults: %H = 5, TT = 0.5 mg/dl, AT = 0.5 mg/dl, and TT/TL = 0.8. Table 5 depicts the ability of the usual critical values to predict an abnormal %H and the summary statistics are as noted. These data support those of Table 2 in pointing out the poor ability of the TT/TL ratio of 0.8 to correctly classify preterm infants. Furthermore, it is noteworthy that in this group of patients little accuracy is gained by using AT rather than TT.

Other investigators (4, 36, 37) have used

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**TABLE 4**

The regression analyses of %H as a function of transformed* tocopherol measures

<table>
<thead>
<tr>
<th>Tocopherol measure</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>Correlation coefficient</th>
<th>Tocopherol measure at %H = 5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT†</td>
<td>-3.78</td>
<td>-1.483</td>
<td>0.68†</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>AT§</td>
<td>-1.199</td>
<td>-4.982</td>
<td>+0.2738</td>
<td>0.69§</td>
<td>0.44</td>
</tr>
<tr>
<td>TT/TL ratio†</td>
<td>-2.16</td>
<td>-0.150</td>
<td>0.59§</td>
<td>1.67</td>
<td></td>
</tr>
</tbody>
</table>

* Transformations: logit %H; log TT; log AT; log TT/TL.
† Model: y = a + bx.
§ Test of significance of regression by F test, p < 0.001.
§ Model: y = a + bx + cx².
TABLE 5
Prediction of %H using usual adult critical values*

<table>
<thead>
<tr>
<th>%H</th>
<th>&lt;=5%</th>
<th>&gt;5%</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
<td>&gt;0.5</td>
<td>155</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>&lt;=0.5</td>
<td>28</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>183</td>
<td>120</td>
</tr>
<tr>
<td>AT</td>
<td>&gt;0.5</td>
<td>147</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>&lt;=0.5</td>
<td>36</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>183</td>
<td>120</td>
</tr>
<tr>
<td>TT/TL ratio</td>
<td>&gt;0.8</td>
<td>178</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>&lt;=0.8</td>
<td>5</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>183</td>
<td>120</td>
</tr>
</tbody>
</table>

Tocopherol measure | % Agreement | % Adjusted agreement | Sensitivity | Specificity | True-positive | True-negative |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
<td>77</td>
<td>84</td>
<td>75</td>
<td>81</td>
<td>90</td>
<td>75</td>
</tr>
<tr>
<td>AT</td>
<td>88</td>
<td>80</td>
<td>84</td>
<td>85</td>
<td>90</td>
<td>81</td>
</tr>
<tr>
<td>TT/TL ratio</td>
<td>76</td>
<td>72</td>
<td>81</td>
<td>83</td>
<td>90</td>
<td>78</td>
</tr>
</tbody>
</table>

* Sensitivity is the percentage of abnormal %H correctly identified. Specificity is the percentage of normal %H correctly identified. True-positive is the percentage of abnormal vitamin E values that had abnormal %H values. True-negative is the percentage of normal vitamin E values that had normal %H values.

other critical values of %H. If we first assume that a %H critical value of 2, 3, or 5% may discriminate for antioxidant status, and then if we choose a critical value of TT, AT, or TT/TL which maximizes the percentage adjusted agreement, modest improvement in the percentage adjustment agreement is achieved for TT and AT with vast improvement in the performance of the new TT/TL critical value as shown in Table 6. In most instances, this improved agreement is achieved only by raising the critical values above the usual adults levels. For instance, at %H = 5, TT increases from 0.50 to 0.64 mg/dl and TT/TL from 0.8 to 1.6; interestingly, AT remains close to 0.50 mg/dl.

Table 7 displays the results of an analysis in which both the %H and the specific tocopherol measure critical values were allowed to change to values which maximized the percentage adjusted agreement. This analysis suggests the optimal %H for plasma tocopherol measures is 3.4% while the TT/TL ratio is optimized at a lower %h of 2.7%. It is interesting to note again that the corresponding AT discriminant value is 0.5 mg/dl, a value that is well established in large population surveys of adults (5, 6). In all of the above analyses, when compared to AT,

TABLE 6
The optimal tocopherol measure critical values for fixed %H of 2, 3, or 5%

<table>
<thead>
<tr>
<th>Critical %H measure</th>
<th>Critical tocopherol measure</th>
<th>% Adjusted agreement</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>True-positive</th>
<th>True-negative</th>
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<tr>
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<td>0.90</td>
<td>68</td>
<td>79</td>
<td>90</td>
<td>75</td>
</tr>
<tr>
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<td></td>
<td>0.54</td>
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<td>84</td>
<td>81</td>
<td>84</td>
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<tr>
<td>TT/TL ratio</td>
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<td>80</td>
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<td>76</td>
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<tr>
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<td>89</td>
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<tr>
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<td>85</td>
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<tr>
<td>TT/TL ratio</td>
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<td>1.9</td>
<td>64</td>
<td>75</td>
<td>90</td>
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</tr>
<tr>
<td>TT</td>
<td></td>
<td>0.64</td>
<td>67</td>
<td>73</td>
<td>93</td>
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</tr>
<tr>
<td>AT</td>
<td></td>
<td>0.47</td>
<td>68</td>
<td>78</td>
<td>90</td>
<td>85</td>
</tr>
<tr>
<td>TT/TL ratio</td>
<td></td>
<td>1.6</td>
<td>60</td>
<td>72</td>
<td>87</td>
<td>83</td>
</tr>
</tbody>
</table>
Comparisons of correlative results from regression analyses
to tocopherol are necessary to maximize the percentage adjusted agreement. This would support the suggestion that non-α isomers of tocopherol are less active as antioxidants and higher plasma concentrations of these isomers are needed to achieve antioxidant activity equal to AT (21, 38).

Blood samples in which the hemolysis assay was performed both with and without added glucose (0.01 M) are analyzed in Table 8. It is notable that although added glucose markedly lowers the critical values of the tocopherol measures (36, 39), both the correlation coefficients for the raw data values and those obtained on transformed data are much worse in the added-glucose system.

Discussion

As an indicator of vitamin E status, each measure of tocopherol status has advantages and disadvantages. The peroxide-induced in vitro hemolysis assay is thought to reflect general erythrocyte antioxidant status with tocopherol preeminent among antioxidants, but the assay is quite sensitive to a variety of test parameters requiring strict technique (24) and represents only a crude measure of tissue antioxidant reserve. More frequently, plasma or serum levels of TT or AT have been assayed but in view of the proposed role for tocopherol at the tissue membrane level, the value of circulating tocopherol measures has been questioned (40). Because of a described correlation and an inferred dependence of plasma tocopherol on plasma lipid levels, a ratio of TT or AT to plasma β-lipoprotein, cholesterol, or total lipid has been advocated in recent years as a more precise assessment of tocopherol status (8, 25, 36).

These technological difficulties aside, the clinical production and assessment of a nutritional deficiency in fragile preterm low birth weight infants poses philosophical, ethical, and pragmatic problems. Because preterm low birth weight infants are abnormal by their very existence ex utero the concept of normality does not apply in the usual clinical sense to this uniformly abnormal population. Furthermore, the experimental creation of a clinically apparent tocopherol deficiency state in a developing preterm infant by withholding vitamin E may result in irreversible adverse effects (41) which would help delineate the disease state but at an ethically unacceptable price.

When plasma tocopherol levels have been related to plasma levels of total lipids (8), cholesterol (8, 25), and β-lipoprotein (36), such data have generally been acquired in enterally nourished individuals receiving mixed diets in which the tocopherol and lipid intakes may be assumed to have been stable during the study period. Furthermore, a few studies (42, 43) have collated cross-sectional data from widely disparate patient groups (eg, infant, nonpregnant adults, and

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**TABLE 7**
The optimal %H and tocopherol measure critical values

<table>
<thead>
<tr>
<th></th>
<th>Critical %H measure</th>
<th>Critical tocopherol measure</th>
<th>% Adjusted agreement</th>
<th>Sensitivity</th>
<th>Specitivity</th>
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<th>True-negative</th>
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<td>65</td>
<td>75</td>
<td>90</td>
<td>90</td>
<td>76</td>
</tr>
</tbody>
</table>

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**TABLE 8**
A comparison of %H* correlation with tocopherol measures with and without 0.01 M glucose

<table>
<thead>
<tr>
<th>Correlations with tocopherol measures</th>
<th>r, all days</th>
<th>0.00 M</th>
<th>0.01 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log (TT)</td>
<td>-0.76</td>
<td>-0.62</td>
<td></td>
</tr>
<tr>
<td>Log (AT)</td>
<td>-0.76</td>
<td>-0.62</td>
<td></td>
</tr>
<tr>
<td>Log (TT/TL ratio)</td>
<td>-0.71</td>
<td>-0.52</td>
<td></td>
</tr>
</tbody>
</table>

Comparisons of correlative results from regression analyses

<table>
<thead>
<tr>
<th>r</th>
<th>Critical values</th>
<th>0.00 M</th>
<th>0.01 M</th>
<th>0.00 M</th>
<th>0.01 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log (TT)</td>
<td>0.75</td>
<td>0.62</td>
<td>0.63</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>Log (AT)</td>
<td>0.77</td>
<td>0.66</td>
<td>0.48</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>Log (TT/TL ratio)</td>
<td>0.71</td>
<td>0.52</td>
<td>2.1</td>
<td>0.94</td>
<td></td>
</tr>
</tbody>
</table>

* %H transformed to logit scale.
† Using same models as in Table 4, with new coefficients.
pregnant females) to extend this observed relationship across decades of life, implying the relationship is stable. However, when one examines the TL relationship of our entire group of patients (Fig 1), it is clear that high plasma tocopherol levels can distort the linear correlation in a fashion suggesting that plasma lipids can be "loaded" with tocopherol beyond the ratios usually observed in healthy individuals. This may suggest that the heretofore observed relationship is coincidental; ie, correlated in a non-specific reflection of overall nutritional status. The increases or decreases in both plasma tocopherol and plasma lipid that have been previously observed in disease states (25) may then be as a result of as yet undescribed alterations common to both measures rather than necessarily any specific change in a plasma transport lipid fraction that then results in a change in plasma tocopherol. In tocopherol-supplemented groups the relationship between iT and TL may be best described with logarithmic transformation of each measure.

It is to be noted that several of our infants were receiving intravenous Intralipid or artificial formulas, both known to contain high levels of $\beta + \gamma$ and $\delta$-tocopherol isomers (21). Our observation that "normal" %H is achieved at AT levels of 0.50 mg/dl but the necessary TT level is 0.64 mg/dl supports the relative impotency in vivo of non-AT isomers. In such an instance, a higher TT/TL ratio would be needed to compensate for the less active isomers when they comprise a major fraction of intake. Indeed, we have identified eight infants (21) who had %H > 5% despite TT > 0.50 mg/dl in whom relatively high levels of $\beta + \gamma$-tocopherol (0.101 to 0.362 mg/dl) were associated with low levels of AT (< 0.50 mg/dl).

As noted earlier, prematurely born low birth weight infants are abnormal by their very existence, producing philosophical and practical difficulties for defining "normal." Historically, two resolutions of this dilemma have been attempted: 1) descriptions of new ranges of normal or "reference values," or 2) efforts to define new discriminants of a disease state. The former response is easily obtained but of practical usefulness only if one assumes no harm is associated with these age-adjusted reference values. We have attempted the latter, rejecting the notion that these lower levels of tocopherol measures found in preterm infants merely represent a new set of age-adjusted reference values. This approach is supported by the observed persistently high %H until near-adult levels of TT or AT are attained.

In order to pursue a definition of the disease state of "tocopherol-deficient" premature infants, we first made the assumption that an elevated in vitro peroxide-induced hemolysis test reflected, albeit crudely, the existence of some potentially injurious situation (one may postulate relatively low oxidant-stress in utero with an appropriately low oxidant-stress defense that is then rendered inadequate by a premature birth into a high oxidant-stress state ex utero). In view of data indicating a compensatory protective role for tocopherol in the face of diminished plasma concentrations of other antioxidants (44), we made the further assumption that tocopherol occupies a central and preeminent antioxidant role among all naturally occurring antioxidants particularly for membrane lipids. Thus, it follows that one may approximate the critical values for "normalcy" of tocopherol measures by using the peroxide hemolysis assay as the measure of whether a deficiency state exists or not. Figure 3 clearly depicts that an imperfect threshold relationship appears to exist between TT values and %H. This general relationship to %H holds for AT and iT/TL as well (data not shown), and was previously reported by Farrell, et al (25) in a study of patients with cystic fibrosis and by Bieri and Poukka (26) in a study of rats. Logistic transformation of %H as indicated and of TT, AT, or TT/TL to the respective logarithm markedly improves the apparent linear relationship (Fig 5) and permits analysis by standard regression techniques. This procedure has the effect of more heavily weighting the central values to improve description of the inflection point which defines that critical value. It is noteworthy that the critical values for TT and AT determined in this fashion (Table 4) are very close to the 0.5 mg/dl proposed for adults. Of further interest is the much higher TT/TL ratio predicted by these data. Horwitt et al (8)
recommended a discriminant ratio of 0.8 for adults while Farrell et al (22) suggested that a ratio as low as 0.6 might apply to children.

In this analysis the correlation coefficient for TT/TL with %H is much lower than for either TT or AT alone. This relationship is not improved by restricting the analysis to those data points in which TT is less than the proposed upper limit of normal for adults of 1.6mg/dl (1). It may be that TT/TL is an appropriate index in a steady state or when a malnourishment condition is being slowly established as in older children with malabsorption (25), but not in the rapidly changing nutritional state of the small premature infant.

It may be soundly argued that the in vitro peroxide-induced hemolysis assay is only a very crude reference for tissue antioxidant reserve, and that because premature infants have erythrocyte polyunsaturated fat contents higher than adults, one should be suspicious of any definition of deficiency based on this assay alone. We are, indeed, suspect. Yet because of the practical difficulty in obtaining approval and consent for fat biopsy or sural nerve biopsy from such patients, we are restricted to analyses of these data. From these analyses, we conclude that true vitamin E deficiency and not just low plasma tocopherol concentrations exist in the majority of preterm low birth weight infants at birth. Furthermore, the tocopherol measures that define the deficient state in our laboratory are a plasma TT of less than 0.64 mg/dl, an AT of less than 0.50 mg/dl, or a TT/TL ratio of less than 1.9. More generally, we propose that the usual adult critical value applies for plasma AT concentrations, but that utilization of TT should include isomeric assessment.

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