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**Evaluation Of The Erythrocyte Malondialdehyde (MDA) Release Assay**

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EVALUATION OF THE ERYTHROCYTE MALONDIALDEHYDE (MDA) RELEASE ASSAY

In the past, vitamin E status has most commonly been measured utilizing a static index such as the plasma concentration. However, since vitamin E circulates with plasma lipids, the plasma vitamin E concentration is considered to be unreliable in some clinical situations. For example, in patients with chronic cholestasis or hyperlipidemia, it has been suggested that the increased lipids in these disorders cause vitamin E to partition from the RBC membrane and other tissues. Thus, a falsely elevated plasma vitamin E level is observed and is not reflective of total body vitamin E.

We evaluated a functional approach for assessing vitamin E status which circumvents this partitioning problem. In evaluating the MDA release assay we performed anticoagulant, stability, and precision studies. For clinical studies, we examined vitamin E sufficient adults, vitamin E sufficient and deficient children, and hyperlipidemic males. Results were compared with several static indices including plasma vitamin E concentration and plasma total lipid concentration. We have refined the MDA release assay to the point where it can be a useful clinical laboratory procedure.

Selected References

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Captain, United States Air Force

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by

JANICE F. GASKA

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Approved by

Robert F. Laskin
(Chairperson of Supervisory Committee)

[Signatures]

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Department of Laboratory Medicine

Date 11 June 1987
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Tables</td>
<td>iv</td>
</tr>
<tr>
<td>List of Figures</td>
<td>v</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>vi</td>
</tr>
<tr>
<td>Chapter 1: Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>1</td>
</tr>
<tr>
<td>Distribution and Dietary Intake</td>
<td>4</td>
</tr>
<tr>
<td>Absorption, Storage, and Transport</td>
<td>4</td>
</tr>
<tr>
<td>Physiologic and Therapeutic Roles of Vitamin E</td>
<td>6</td>
</tr>
<tr>
<td>Assessment of Nutritional Status</td>
<td>12</td>
</tr>
<tr>
<td>Review of Static Assays</td>
<td>14</td>
</tr>
<tr>
<td>Review of Functional Assays</td>
<td>19</td>
</tr>
<tr>
<td>Scope of This Research</td>
<td>21</td>
</tr>
<tr>
<td>Chapter 2: Materials and Methods</td>
<td>24</td>
</tr>
<tr>
<td>Reagents</td>
<td>24</td>
</tr>
<tr>
<td>Procedure</td>
<td>25</td>
</tr>
<tr>
<td>Determination of Optimum Hydrogen Peroxide Concentrations</td>
<td>28</td>
</tr>
<tr>
<td>Vitamin E Assay</td>
<td>29</td>
</tr>
<tr>
<td>Cholesterol Assay</td>
<td>29</td>
</tr>
<tr>
<td>Triglyceride Assay</td>
<td>30</td>
</tr>
<tr>
<td>Total Lipids Assay</td>
<td>30</td>
</tr>
<tr>
<td>Chapter 3: Results and Discussion</td>
<td>31</td>
</tr>
<tr>
<td>Standard Curve</td>
<td>31</td>
</tr>
<tr>
<td>Determination of Optimum Hydrogen Peroxide Concentrations</td>
<td>31</td>
</tr>
</tbody>
</table>
Anticoagulant Studies...........................................34
Stability Studies..................................................37
Precision Studies..................................................40
Clinical Studies..................................................42
Chapter 4: Conclusions.........................................61
Further Prospective Studies....................................62
List of References..............................................65
LIST OF TABLES

<table>
<thead>
<tr>
<th>Number</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Standard Curve Data</td>
<td>33</td>
</tr>
<tr>
<td>2</td>
<td>Anticoagulant Studies</td>
<td>38</td>
</tr>
<tr>
<td>3</td>
<td>Stability Studies</td>
<td>39</td>
</tr>
<tr>
<td>4</td>
<td>Precision Studies</td>
<td>41</td>
</tr>
<tr>
<td>5</td>
<td>Group Comparisons</td>
<td>45</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Number</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Alpha-Tocopherol</td>
<td>3</td>
</tr>
<tr>
<td>2.</td>
<td>Standard Curve</td>
<td>32</td>
</tr>
<tr>
<td>3.</td>
<td>Optimum Hydrogen Peroxide Concentrations (without azide)</td>
<td>35</td>
</tr>
<tr>
<td>4.</td>
<td>Optimum Hydrogen Peroxide Concentrations (with azide)</td>
<td>36</td>
</tr>
<tr>
<td>5.</td>
<td>% MDA vs. Vitamin E</td>
<td>48</td>
</tr>
<tr>
<td>6.</td>
<td>% MDA vs. Vitamin E (Control)</td>
<td>50</td>
</tr>
<tr>
<td>7.</td>
<td>% MDA vs. Vitamin E (CHMC)</td>
<td>51</td>
</tr>
<tr>
<td>8.</td>
<td>% MDA vs. Vitamin E (HLM)</td>
<td>53</td>
</tr>
<tr>
<td>9.</td>
<td>Vitamin E vs. Total Lipids</td>
<td>55</td>
</tr>
<tr>
<td>10.</td>
<td>% MDA vs. Total Lipids</td>
<td>57</td>
</tr>
<tr>
<td>11.</td>
<td>Vitamin E vs. Cholesterol</td>
<td>58</td>
</tr>
<tr>
<td>12.</td>
<td>% MDA vs. Cholesterol</td>
<td>59</td>
</tr>
</tbody>
</table>
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>E/TL ratio</td>
<td>vitamin E/total lipid ratio</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>MDA</td>
<td>malondialdehyde</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acids</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>RDA</td>
<td>recommended dietary allowance</td>
</tr>
<tr>
<td>RLF</td>
<td>retrolental fibroplasia</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
</tbody>
</table>
Vitamin E

Vitamin E, one of the fat soluble vitamins, was discovered by Evans and Bishop in 1922 (1). They observed that female rats had normal early stages of pregnancy when deprived of vegetable oils, but later uniformly aborted. Evans and Bishop found that vitamins A and D did not help prevent the problem but actually appeared to worsen it, whereas the addition of wheat germ oil to the diet completely prevented abortion. Thus, they found that the wheat germ oil was essential for reproduction in the rat. Sure confirmed these studies and called the wheat germ factor vitamin E because it was different from either vitamin A or D and was fat soluble (2). Although a similar function of preventing abortion has not been found in humans, recent research has shown vitamin E to have an essential role in animal and human nutrition as well as therapy for several human clinical disorders (3).

Vitamin E represents a group of several naturally occurring tocopherols designated as alpha-, beta-, gamma-, delta-, eta-, and zeta-tocopherol. The term tocopherol comes from the Greek words, tocos (offspring), pheein (to bear), and oel (to signify an alcohol) (3).
Alpha-tocopherol has the maximum biologic activity and comprises approximately 87% of the total tocopherol concentration (4). Thus, alpha-tocopherol is the tocopherol that is commonly measured in the clinical laboratory. The basic structure of vitamin E, shown in Figure 1, consists of an hydroxylated ring system which is involved in free radical reactions, and an isoprenoid side chain which accounts for the vitamin being soluble in lipids.

The most important known biologic role of vitamin E is as an antioxidant (3,5-7). Free radicals that are generated by normal metabolic processes or from toxic compounds taken into the body (e.g., ozone) attack polyunsaturated fatty acids in cellular membranes. This latter reaction, in turn, causes peroxidative decomposition of membrane lipids, leading to cellular damage. Vitamin E is the major chain-breaking antioxidant in blood and acts by trapping the chain-propagating free radicals. In other words, the oxidation of unsaturated fatty acids can be inhibited dramatically by the presence of vitamin E.

Vitamin E is bound to the cellular and subcellular membranes by specific physicochemical interactions between its phytol side chain and the fatty acyl chain of the membrane polyunsaturated phospholipids, especially those derived from arachidonic acid. In this way, the architecture of the membranes is stabilized. Therefore,
FIGURE 1

Alpha-Tocopherol
when vitamin E deficiency is present, red cell membranes become more easily susceptible to hemolysis.

**Distribution and Dietary Intake**

Vitamin E is one of the most widely distributed vitamins in foods, with the richest sources being vegetable oils such as soybean, corn, cottonseed, and safflower oils and products made from them (6). Vitamin E is also found in grain products, meats, poultry, and fish (5). The recommended dietary allowance (RDA) of alpha-tocopherol is 7-13 mg or 10-20 IU (8). Studies have shown that as the intake of polyunsaturated fatty acids increases, the daily requirement for vitamin E increases also (5,9). The average daily intake with a balanced adult diet ranges from 7-9 mg (11-14 IU) and is considered to be adequate in healthy individuals. Even though vitamin E is the least toxic of the fat soluble vitamins, doses above 1200 IU/day can cause toxic symptoms such as nausea, diarrhea, intestinal cramps, skin reactions, myopathy, and gonadal dysfunction and can alter vitamin K's function as an anticoagulant (10).

**Absorption, Storage, and Transport**

Since vitamin E is fat soluble, absorption is
dependent upon many of the same mechanisms that regulate
the uptake of lipids. It has been shown that bile is
essential for absorption and for maximal absorption,
incorporation of the vitamin into mixed micelles is also
necessary (11,12). A study in human beings has shown an
intestinal absorption of about 25% when the vitamin was
measured in the lymph (13). Most of the absorbed vitamin
is carried in the chylomicron fraction. It has been
suggested that this low intestinal absorption of tocopherol
might account for the vitamin E deficiency associated with
a diet enriched with polyunsaturated fatty acids (13).

After tocopherol is transported to the vascular
circulation in chylomicrons, it is then carried mainly in
low density lipoproteins (LDL) and/or high density
lipoproteins (HDL) (14,15). Circulating tocopherol is
accumulated slowly by the tissues. There is no single
storage organ for vitamin E but in terms of absolute
amounts, adipose tissue, liver, and muscle contain most of
the body's tocopherol.

Even though most American diets are now two to three
times richer in gamma-tocopherol than alpha-tocopherol, the
concentration of gamma in plasma and tissue is
approximately one-tenth that of alpha (16-18). Behrens and
Madere (19) as well as Handelman et al (20) have proposed
that the mechanism regulating the absorption, plasma
transport, and tissue uptake is determined by specific
carriers and/or binding sites for alpha-tocopherol. However, several other investigators postulate that the concentration of total lipids in plasma or tissues is the determining factor for the plasma tocopherol concentration (21,22). Even if lipids control the amount of tocopherol in plasma or tissues, this does not explain why gamma-tocopherol concentration is lower in plasma and tissues in spite of being more abundant in the diet. This problem is currently under investigation.

Physiologic and Therapeutic Roles of Vitamin E

Since the discovery of vitamin E 65 years ago, there has been much debate over the exact physiologic and therapeutic roles of the vitamin. Even the human dietary requirement for vitamin E is in doubt. Research over the past few years has focused on defining these roles more clearly but there are still many unanswered questions. One thing is known, the antioxidant role of vitamin E, by itself, cannot account for the wide range of manifestations associated with vitamin E deficiency.

Vitamin E deficiency states in various animal species exhibit such findings as painless muscle atrophy and proximal weakness. The associated histological changes are consistent with degenerative changes in muscle and spinal cord. The severity of muscle and cord lesions
correlates well with the maturity of the animal and the period of vitamin E deficiency (23). Some researchers have suggested that the progress of neurologic lesions can be either arrested or reversed by vitamin E replacement therapy (24,25). In human beings, Satel et al (26) have suggested that behavioral abnormalities in patients with Alagille syndrome (arteriohepatic dysplasia) are associated with vitamin E deficiency. The observed behavioral abnormalities included substance abuse, impulsivity, conduct disorder, and antisocial behavior. However, the association between vitamin E deficiency and behavioral abnormalities in patients with other chronic cholestatic syndromes has not been confirmed by others.

In man, abetalipoproteinaemia was the first disorder associated with the neurologic syndrome caused by vitamin E deficiency (27). The spinocerebellar syndrome in these patients is characterized by a progressive neurologic syndrome comprising cerebellar ataxia, posterolateral column dysfunction, peripheral neuropathy and retinal degeneration. In abetalipoproteinaemia, there is a failure to synthesize chylomicrons because of the total absence of beta-lipoprotein. This results in the malabsorption of lipids and the fat-soluble vitamins K, A, D, and E. Many other chronic disorders of fat malabsorption, such as congenital biliary atresia, cystic fibrosis, and blind loop syndrome, are also associated with vitamin E deficiency.
Neurologic manifestations in these disorders are similar to those observed with abetalipoproteinemia (3,28,29). From these observations, one can conclude that vitamin E is essential for the normal structure and function of neurons.

Vitamin E responsive hemolytic anemia, which is observed in premature infants, has been suggested by Moudgil and Narang (3) to be the only human disorder that can be strongly linked to vitamin E deficiency. This anemia is characterized by a low plasma concentration of vitamin E and an increased susceptibility of red blood cells to peroxide hemolysis. Laboratory tests reveal reticulocytosis, thrombocytosis, and a decreased hemoglobin. Clinical symptoms include edema of the legs, external genitalia and eyelids, watery nasal discharge, tachypnea and restlessness. The condition is self limiting and requires treatment with vitamin E only during the first three months of life. However, some investigators have been unable to confirm the beneficial role of vitamin E in this condition (30,31).

Numerous factors may contribute to the vitamin E deficient status of the small premature infant. First, these infants lack the fat depots in which the vitamin is normally stored. Secondly, they are usually fed artificial diets in which the fat is supplied by polyunsaturated fatty acids (PUFAs), namely, linoleic acid. Furthermore, the amount of PUFAs in infant formulas is normally in excess of
that in breast milk so more of the vitamin is needed to satisfy the high fat intake. Finally, the premature infants may have increased requirements for vitamin E because of rapid growth (5).

Another condition involving premature infants is retrolental fibroplasia (RLF). RLF is a retinal disease that affects the growth of immature retinal blood vessels and may lead to blindness. The disease is of unknown etiology (32). One factor that may contribute to the development of RLF is the exposure of retinal blood vessels to a hyperoxic environment from oxygen therapy. There also seems to be a relationship between vitamin E deficiency and the development of RLF (33).

Many studies in the past few years have documented a beneficial role for vitamin E in reducing the incidence and severity of RLF in low birth weight infants (34-37). The proposed mechanism of action of vitamin E in RLF is the inhibition of vasoconstriction and platelet aggregation resulting from hyperoxia via an effect on the antithrombotic agent, prostacyclin (38). Furthermore, free oxygen radicals seem to increase the gap junctions between embryonic spindle cells (the embryonic precursors of inner retinal capillary endothelial cells). Widening of the gap junction is followed by the pathological process, spindle cell proliferation, and finally neovascularization (39). The antioxidant action of vitamin E may help suppress the
sequence described above which leads to the development of
RLF.

A second condition associated with the delivery of
high concentrations of oxygen is bronchopulmonary
dysplasia. Studies have shown that animals fed a vitamin E
deficient diet are more susceptible to oxygen toxicity
(40,41). Additionally, it has been shown that vitamin E
protects the lungs of animals from oxygen toxicity (42).
Studies of infants have proven to be inconclusive and
further research is needed to evaluate the protective
efficacy of vitamin E in bronchopulmonary dysplasia
(43-45).

Association of vitamin E with cardiac and circulatory
diseases has received wide attention since the claim was
made in 1948 that alpha-tocopherol supplements were
beneficial in patients with angina (46). Several authors
have investigated this purported effect and have concluded
that vitamin E has not been proven to have a beneficial
role in cardiac disorders (47-49). Nevertheless, vitamin E
seems to play a role in inhibiting platelet aggregation by
inhibiting the formation of platelet-aggregating
endoperoxide intermediates of prostaglandin biosynthesis
(5). There is also some evidence that vitamin E is
effective in the treatment of intermittent claudication but
more research is needed to determine the role, if any, of
tocopherol in this setting (49,50).
Recent epidemiological and animal data suggest that selenium and vitamins E, C, and A (including its provitamin beta-carotene) act as anticarcinogens; hypothetically they alter cancer incidence, differentiation, and/or growth (9,51). All of these nutrients act synergistically as antioxidants and therefore protect cell membranes from auto-oxidative damage. Selenium in the active site of glutathione peroxidase reduces hydroperoxides and beta-carotene traps free radicals and singlet oxygen. Vitamin E is the primary antioxidant that prevents lipid peroxidation by trapping free radicals while vitamin C is a water-soluble chain-breaking antioxidant (9). Once vitamin E traps the oxygen radicals it is consumed and must be regenerated before it is capable of functioning again as an antioxidant. Vitamin C and other reducing agents such as glutathione, NADH, and cysteine can reduce the vitamin E radical to regenerate vitamin E (52,53).

Both vitamins C and E inhibit nitrosation, the conversion of nitrite to nitrosoamines and nitrosoureas. This blocking effect on nitrosation implicates these vitamins as protective agents against activated oxygen (superoxide ion, hydroxyl radical, etc.). For example, a study with mice revealed that when vitamin E was administered topically or orally, chemically induced skin tumors and sarcomas were decreased (54). With the addition of 400 mg of vitamin E to the diet, fecal mutagens produced
endogenously in human beings decreased by 2 to 26% (55). Menkes et al (51) recently suggested that low serum vitamin E levels are related to an increased incidence of all types of lung cancer in human beings. Future research in this area should prove to be very worthwhile.

Assessment of Nutritional Status

The conventional approaches to nutritional status assessment have involved utilizing static indices. This type of index measures the plasma or serum concentration at a given point in time. An alternative to the static index is the functional index, which is a test of a physiologic or behavioral function that is dependent upon a given nutrient(s). Vitamin E, like most nutrients, can best be assessed by utilizing a functional test as explained below.

The purpose of nutritional assessment is to determine whether body tissues have an adequate supply of the nutrient of interest to sustain normal metabolism. Under appropriate conditions, static indices can provide useful information. However, static indices are known to have many pitfalls and/or limitations (56). Theoretically, at least, stores might become depleted before the decrease is reflected in circulating concentrations of the vitamin. Another problem concerning tocopherol is that the binding capacity of lipoproteins for tocopherol determines the
circulating level of the vitamin. This can complicate the interpretation of results for patients with chronic cholestasis who may have plasma tocopherol levels in the reference range but in reality are vitamin E deficient. The reason for the normal plasma tocopherol in this setting, is that the increased plasma lipids in chronic cholestasis may cause tocopherol to partition from the red blood cell membrane into the plasma (57). This effect is also seen in patients with diabetes mellitus, hypothyroidism, or primary hyperlipidemia (15,21). Thus, increased plasma lipid concentrations can falsely elevate the plasma vitamin E concentration and reduce the antioxidant effect on RBCs and other tissues. Finally, the sample such as blood cells or plasma may only represent active (target) tissue while storage pools such as liver or muscle tissue may differ from plasma levels but are inaccessible except by biopsy.

On the other hand, measurement of the functional index of nutrient status avoids the previously mentioned problems. Perhaps the original functional index of nutritional status was growth since the growth pattern of a child is sensitive to changes in nutrient concentrations. However, growth is more of an indirect approach. In 1913, Hess introduced the first formal, standardized test that could be called a functional index (58). The Hess test
detected subclinical scurvy by enumeration of capillary petechiae under increased venous pressure.

Review of Static Assays

Vitamin E status has most often been assessed by measuring the vitamin level in the serum or plasma. Alpha-tocopherol is the predominant form in the plasma as well as in the red blood cell and is the form that is commonly measured clinically. Levels of alpha-tocopherol in healthy subjects have not been well established but are in the range of 5-20 mg/L in adults. Children have slightly lower values. One of the better methods for measuring either plasma or red blood cell alpha-tocopherol is the Bieri method which utilizes high pressure liquid chromatography (59).

In recent years, several investigators have observed that the plasma level is not reflective of total body status of vitamin E levels (21,60,61). In a clinical situation, low levels of plasma vitamin E more often are due to redistribution rather than depletion and thus, low plasma levels may not reflect true deficiency at tissue level (62). It is also well established that plasma tocopherol levels fluctuate in relation to plasma lipids (15,21,63). In addition, both plasma cholesterol and triglyceride levels may influence alpha-tocopherol
concentration in the blood and therefore, concentrations in other tissues (64). Thus, the plasma level of vitamin E can be considered to be unreliable. For these reasons, Horwitt and others feel that the tocopherol/total lipids (E/TL) ratio is a more reliable index of total vitamin E status (21,22,65). They have suggested that 0.8 mg tocopherol/g of plasma lipid is the lower limit of adequate vitamin E levels in adults. However, the reported reference range is different in different studies (66-68).

More recently, the gamma-tocopherol to alpha-tocopherol ratio has been thought to provide a much more reliable index of compliance to alpha-tocopherol supplementation (20). Behrens and Macare (69) and Handelman et al (20) observed that the plasma gamma-tocopherol is substantially decreased when the plasma alpha-tocopherol is substantially increased by oral alpha-tocopherol supplementation. Similarly, subjects with an increased gamma-tocopherol tend to have a decreased alpha-tocopherol. Thus, it would seem that the gamma to alpha ratio would be more sensitive to changes in alpha-tocopherol. Bieri found that the biologic activity of gamma-tocopherol was approximately one-tenth of alpha-tocopherol activity (17). He also found that gamma-tocopherol disappears more quickly from the circulation than does alpha-tocopherol. It seems that gamma-tocopherol is initially taken up by tissues as
readily as is alpha-tocopherol but gamma turns over faster. The mechanism for this difference in turnover is unknown (17).

In 1981, Lehmann reported that platelet tocopherol was a good indicator of vitamin E nutritional status in rats because of its sensitivity to change in vitamin E intake (70). Vatassery et al (71) also found a high degree of correlation between platelet tocopherol and plasma tocopherol/lipid ratio. Unfortunately, routine measurement of platelet vitamin E levels using this method appears to be less than ideal because of the large amount of blood required (10 mL or more) and the complexity of preparing the platelets (72).

Red blood cell (RBC) tocopherol is yet another blood component that has been utilized to assess vitamin E status. RBC tocopherol has been found to be closely correlated to platelet tocopherol and tocopherol/total lipid ratio (73,74). Alpha-tocopherol has been shown to exchange rapidly between plasma and red cells, reaching equilibrium in 6-8 hours (75). The majority of tocopherol found in the RBC is localized in the membrane (76). Kitabchi et al (77) have suggested that human erythrocyte membranes have specific, saturable binding sites for D-alpha-tocopherol. These binding sites are at least partly protein in nature and may be of physiologic significance in protecting RBCs from hemolytic damage. Due
to earlier methodological difficulties, RBC tocopherol has been measured only in recent years.

Initially, it was thought that the tocopherol in erythrocytes and plasma were directly correlated. However, later reports have shown that they are not as related as previously thought (78-80). In fact, several authors have reported that as plasma lipids increase, e.g. in hyperlipemia, there is an inverse relationship between the tocopherol in RBCs and plasma, i.e. the RBC level decreases while the plasma level increases (78,80,81). In addition, Farrell observed that a decrease in RBC tocopherol concentration was paralleled by a decrease in tissue tocopherol concentration (heart and muscle) in patients with cystic fibrosis (22). Therefore, RBC tocopherol may indeed be more accurate in assessing the nutritional status of vitamin E.

Since tocopherol functions at the membrane level, Mino (82) studied rats to see how well RBC tocopherol concentrations compared to tissue levels. He found that changes in RBC tocopherol concentrations were similar to changes in liver and liver subcellular fractions, especially mitochondria, microsomes, and nuclei. However, it is obvious that the ability to obtain similar convincing tissue tocopherol correlations with RBC tocopherol from human beings is limited. More recently, Mino (83) observed a significant decrease not only in the RBC tocopherol level
but also in the mitochondria and microsomes of the liver in rats with increased plasma tocopherol accompanying hyperlipemia.

Haddad placed hyperlipidemic men on low fat diets and found a decreased plasma tocopherol with decreased lipids but an increased RBC tocopherol (84). This increase in RBC tocopherol suggested an apparent redistribution of vitamin E between plasma lipoproteins and the RBC membrane. Whether the increased RBC content of vitamin E serves as a temporary pool or whether the higher levels are maintained over longer periods of time requires further study.

Peripheral white blood cells (leukocytes) may be yet another component of blood that can be utilized as a static assay for vitamin E assessment. These cells have a rapid turnover and can be tested for response to specific nutritional changes. In a rat study by Omaya (63) in 1986, leukocyte tocopherol was found to closely reflect liver tocopherol. In fact, leukocyte tocopherol correlated better to liver tocopherol than did RBC tocopherol. Logarithmic transformed data of leukocytes and/or platelet vitamin E correlated the best with liver vitamin E changes during depletion. One problem with measuring leukocyte tocopherol is obtaining adequate cell separation without some platelet and leukocyte cross contamination especially when using limited amounts of blood (63).
Review of Functional Assays

The next major breakthrough in functional nutrient assessment after the Hess test, was in the 1930s when the association between impaired dark adaptation response and early vitamin A deficiency was observed. Later, vitamin E deficiency was also found to be related to impaired dark adaptation response.

A test which is more quantitative, the hydrogen peroxide hemolysis test has been used to estimate vitamin E deficiency in human beings for several years (85-87). This assay has often been used for assessing vitamin E deficiency in premature infants. Vitamin E deficiency in human beings is associated with an increased susceptibility of red blood cells to in vitro hydrogen peroxide induced hemolysis. This susceptibility of erythrocytes to oxidative hemolysis is influenced by at least two variables: the nature of the lipids found in the stroma and the quantity of tocopherol attached to the erythrocytes (88). Brin et al (89) have reported that the fatty acid composition and the specific phospholipid structure in a membrane are also important in determining whether the structural integrity will be disrupted in the absence of vitamin E. One advantage of the hemolysis test is that it measures the biologically active portion of total tocopherol (85). More importantly, however, is the disadvantage that the assay
has been found to be difficult to reproduce (86,87).

The most recently developed functional index of vitamin E status is the erythrocyte malondialdehyde (MDA) release in vitro assay (90). This assay is loosely based on the hydrogen peroxide hemolysis test. Erythrocytes are incubated with hydrogen peroxide, resulting in the oxidation of polyunsaturated fatty acids in the RBC membranes. The oxidation products formed include malondialdehyde and other hydrocarbons. The MDA is then measured as the thiobarbituric acid derivative. Since vitamin E aids in preventing oxidation, the quantity of MDA that is formed increases when the amount of vitamin E is decreased.

To provide a more sensitive assay, Cynamon, Isenberg, and Nguyen carried out the hydrogen peroxide incubations not only with sodium azide as others had done earlier but also without sodium azide (90,91). In this assay, sodium azide inhibits erythrocyte catalase, which is an enzyme that catalyzes the reaction \( 2\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}_2\text{O} \). Therefore, in experiments with sodium azide, the hydrogen peroxide is stabilized and is allowed to completely react with the polyunsaturated fatty acids and form MDA. On the other hand, in experiments without sodium azide, the hydrogen peroxide is destroyed quickly by catalase and not as much MDA is formed. The MDA release with catalase inhibition is viewed as the maximal release of MDA possible and the MDA
without inhibition as a reflection of the erythrocyte membrane antioxidant protection. The results are then expressed as a fractional or percent maximal release (90).

Cynamon et al (90) found the MDA release with catalase inhibition in vitamin E sufficient subjects quite similar to that in vitamin E deficient subjects. However, the MDA release without catalase inhibition revealed quite different results between the two groups. Erythrocytes from vitamin E sufficient subjects exhibited minimal to no detectable release of MDA while erythrocytes from vitamin E deficient subjects exhibited release from 40-252 nmol/mL RBCs (90). This increased MDA release in vitamin E deficient subjects is due to the decrease in vitamin E, and therefore, the decreased antioxidant protection of the RBC membranes.

Scope of This Research

The assessment of vitamin E status has recently become more important as the diverse roles of vitamin E in human nutrition and its therapeutic efficacy have become better defined. For these reasons and because of continuing vitamin E research, an accurate method for measuring vitamin E is important. Vitamin E can no longer be considered a vitamin in search of a disease.
In the past, vitamin E status has most frequently been measured utilizing a static index such as the plasma concentration. However, several investigators feel that the plasma vitamin E level is an unreliable index of vitamin E nutritional status (21,60,61). As previously mentioned, plasma lipids play an important role in determining the plasma vitamin E concentration. Much research has involved evaluating other static indices for vitamin E status but all have inherent problems such as the volume of blood required and difficulty in the separation of the blood components to be assayed. Therefore, a functional approach for assessing vitamin E status seems to be the better approach.

The work described herein was undertaken to evaluate a recently developed functional assay for vitamin E status. The erythrocyte malondialdehyde (MDA) release assay was developed for potential use in the clinical laboratory to provide a more accurate assessment of vitamin E status than the conventional method of measuring plasma vitamin E concentration. The evaluation of the MDA assay includes the effects of various anticoagulants, the stability of the results from samples assayed at different times after collection, and within-run precision studies. The clinical usefulness of the MDA assay was evaluated by studying vitamin E sufficient adults, vitamin E sufficient and deficient children, and hyperlipidemic males. Results
from the MDA assay were compared with several static indices including the plasma vitamin E concentration and plasma total lipids concentration.
Chapter 2

Materials and Methods

Erythrocyte Malondialdehyde (MDA) Release In Vitro Assay

Reagents

1. Isotonic phosphate buffered saline (PBS), pH 7.4 (Sigma Chemical Co., St. Louis, MO 63178). Preparation: Reconstituted each vial with 1 L of deionized water and stored at 4°C.
2. PBS with sodium azide (Mallinckrodt Inc., Paris, KY 40361). Preparation: 26.0 mg sodium azide/100 mL PBS. Stored at 4°C.
3. Hydrogen peroxide, 30% (Mallinckrodt). Stored at 4°C.
4. Trichloracetic acid (TCA), 28% (Mallinckrodt) in sodium arsenite (J.T. Baker Chemical Co., Philipsburg, NJ 08865). Preparation: 28.0 g TCA/100 mL 0.1 mol/L sodium arsenite. Stored at room temperature and filtered before each use with ashless #42 filter paper (Whatman Inc., Clifton, NJ 07014).
5. 2-Thiobarbituric acid, 1% (Sigma) in sodium hydroxide (Fisher Scientific Co., Fairlawn, NJ 07410). Preparation: 1 g 2-thiobarbituric acid/100 mL of 0.05 mol/L sodium hydroxide. Stored at room temperature and filtered before each use with ashless #42 filter paper (Whatman).
6. Stock standard, 400 nmol/mL. Preparation: Diluted 0.1 mL of 1,1,3,3-tetraethoxypropane (Sigma) to 1 L with deionized water. Stored at 4°C.

Procedure

The MDA release assay was performed following the procedure by Cynamon, Isenberg, and Nguyen (90). After a 10-12 hour fast, samples of peripheral venous blood were collected in tubes containing either heparin or EDTA. The original procedure used citrated blood. In addition, heparin and EDTA samples were assayed in parallel with citrated samples and were also found to provide acceptable results (see Chapter 3 Results and Discussion). Within two hours of collection, all samples were centrifuged at approximately 1400 x g (2000 rpm) for 10 minutes in an IEC Model CL Clinical Centrifuge (International Equipment Co., Needham Hts., MA 02194). After centrifugation, the plasma was separated from the red cells and, if not analyzed the same day, the red cells were stored at 4°C and analyzed within 48 hours of collection.

Two aliquots of 0.3 mL of red cells were each washed once with 10.0 mL of PBS, pH 7.4 and centrifuged at approximately 850 x g (2000 rpm) for 4 minutes in an IEC Model CRU 5000 centrifuge (IEC). From one aliquot of washed red cells, duplicate 5% RBC suspensions were
prepared by adding 0.05 mL packed red cells to 0.95 mL PBS without sodium azide. The second aliquot of washed red cells was used to make duplicate 5% RBC suspensions using PBS with sodium azide as the diluent.

Specimen blanks, in duplicate, were made by adding 0.05 mL of the packed red cells to 1.95 mL PBS. Duplicate reagent blanks each consisted of 2.0 mL of PBS. Frozen aliquots of a high and a low control were thawed immediately before use and run in duplicate. The controls were prepared by adding 0.05 mL of packed red cells to 0.95 mL of two different concentrations of standard to yield a high and low concentration of MDA. Once the controls were thawed 1.0 mL of PBS was added to each tube.

To each 5% RBC suspension that did not contain sodium azide, 1.0 mL of a freshly prepared 3% solution of hydrogen peroxide was added. The hydrogen peroxide solution was prepared using PBS as the diluent. To each 5% RBC suspension that contained sodium azide, 1.0 mL of a freshly prepared 0.75% solution of hydrogen peroxide was added. The determination of the optimal hydrogen peroxide concentration with and without azide will be discussed later. Hydrogen peroxide was not added to the specimen blanks, reagent blanks, or controls. Thus, each tube contained a final volume of 2.0 mL.

All tubes were mixed and immediately incubated for one hour in a 37°C water bath (Precision Scientific Company,
Chicago, IL 60647). Following incubation, 1.0 mL of 28% TCA in sodium arsenite was added to all tubes. The TCA aided in precipitating potentially interfering proteins while the sodium arsenite was used to stabilize the MDA chromogen. Each tube was vortexed at a high speed setting for 10 seconds, left at room temperature for 15 minutes, and vortexed again for 10 seconds. This was done in order to obtain a more clear supernatant, and is a modification of the original procedure.

The tubes were centrifuged at approximately 1800 x g (3000 rpm) for 10 minutes in a Model CRU 5000 centrifuge (IEC). Two mL of the supernatant from each tube were removed and placed into clean 16 x 100 mm glass tubes. Care was taken to avoid pipetting any of the precipitate. One-half mL of 1% 2-thiobarbituric acid in sodium hydroxide was added to all tubes. The tubes were covered with foil and boiled for 15 minutes in a water bath. After the samples were cooled to room temperature, the absorbance at 535 nm was measured using a Gilford 2400-S Spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH 44074).

The MDA concentrations were determined using a standard absorption curve for malondialdehyde that was prepared using 1,1,3,3-tetraethoxypropane (malonaldehyde tetraethyl acetal). A stock standard of 400 nmol/mL was diluted with deionized water to make working standards of
1.0, 6.0, 12.0, and 20.0 nmol/mL concentrations. Two mL each of PBS, working standard, and 28% TCA in sodium arsenite were mixed together and carried through the rest of the procedure beginning after the one hour incubation. Results are reported as the percent of maximal MDA release or percent MDA according to the following equation suggested by Cynamon et al (90):

\[
\frac{\text{MDA release (3\% H}_2\text{O}_2)}{\text{MDA release (0.75\% H}_2\text{O}_2 \text{ plus azide})} = \%\text{MDA}
\]

**Determination of Optimum Hydrogen Peroxide Concentrations**

The following experiments were performed in order to determine the hydrogen peroxide concentrations at which maximum MDA release was observed. Hydrogen peroxide concentrations (v/v) of 0.075%, 0.75%, 1.5%, and 3.0% were added to erythrocyte suspensions incubated with and without sodium azide. The hydrogen peroxide concentrations were determined by using the following equation:

\[
A = \varepsilon c l
\]

where \( A \) is the absorbance at 254 nm, \( \varepsilon \), the molar extinction coefficient at 254 nm for \( \text{H}_2\text{O}_2 \), is 23 (92), \( c \) is the concentration in moles/L, and \( l \) is the path length in cm which is 1 in this study.

The concentration of the stock hydrogen peroxide was determined using a Gilford 2400-S Spectrophotometer and was
approximately 28%. The concentration of hydrogen peroxide was checked bimonthly by making a 1:200 dilution of stock hydrogen peroxide. The reagent blank was deionized water.

**Vitamin E Assay**

All plasma vitamin E levels were measured using a modified high-performance liquid chromatography (HPLC) method published by Bieri (59,93). In this method, vitamin E is extracted from plasma into heptane prior to separation and quantitation by HPLC. Results are reported as mg/L.

**Cholesterol Assay**

Plasma cholesterol levels were measured using a Cobas Bio Analyzer (Roche Analytical Instruments, Nutley, NJ 07110) and a Fastchem Cholesterol kit (Boehringer Mannheim Diagnostics Inc., Indianapolis, IN 46250) (94). All cholesterol esters are split quantitatively into free cholesterol and fatty acids by cholesterol esterase. In the presence of oxygen, free cholesterol is oxidized by cholesterol oxidase to form cholest-4-en-3-one and hydrogen peroxide. Hydrogen peroxide then reacts in the presence of peroxidase with phenol and 4-aminophenazone to form a p-quinone imine dye. The intensity of the color formed is
proportional to the cholesterol concentration. Results are reported as mg/dL.

**Triglyceride Assay**

Plasma triglyceride levels were measured by a Cobas Bio Analyzer (Roche) using a colorimetric test (Behring Diagnostics, La Jolla, CA 92037) (95). Triglycerides, in this assay, undergo lipase hydrolysis, phosphorylation, and oxidation/reduction reactions. The final product is a colored formazan complex which has an absorbance that is proportional to the concentration of triglyceride. Results are reported as mg/dL.

**Total Lipids Assay**

Plasma total lipid concentration was approximated as the sum of two times the cholesterol concentration (mg/dL) plus the triglyceride concentration (mg/dL) as suggested by Lehmann et al (96). Results are reported as mg/dL.
Chapter 3

Results and Discussion

Standard Curve

A typical standard curve of 1,1,3,3-tetraethoxypropane (malonaldehyde tetraethyl acetal) and its least squares regression line are shown in Figure 2. The results of the standard curves run over a period of 5 months were reproducible and the mean $\pm$ standard deviation and coefficient of variation (CV) for each concentration of standard is shown in Table 1. Specimen concentrations (nmol MDA/mL RBC) were determined utilizing the final absorbance readings (specimen absorbance - specimen blank absorbance) in the regression equation and multiplying the result by 20 (the dilution factor). The relatively high CV of the lowest concentration of standard (1.0 nmol/ml) probably reflects imprecision of the low spectrophotometric readings.

Determination of Optimum Hydrogen Peroxide Concentrations

Hydrogen peroxide concentrations were varied in the assay of specimens from two healthy subjects to determine the concentrations to add to produce the maximal MDA release in the presence and absence of sodium azide. The
FIGURE 2
Standard Curve

Absorbance at 535 nm

MDA (nmol/mL)

\[ 0.046X + 0.012 \]
Table 1

Standard Curve Data

<table>
<thead>
<tr>
<th>STANDARD (nmol MDA/mL)</th>
<th>ABSORBANCE at 535 nm MEAN ± SD (n = 11)</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.059 ± 0.009</td>
<td>15%</td>
</tr>
<tr>
<td>6.0</td>
<td>0.280 ± 0.014</td>
<td>5%</td>
</tr>
<tr>
<td>12.0</td>
<td>0.550 ± 0.023</td>
<td>4%</td>
</tr>
<tr>
<td>20.0</td>
<td>0.928 ± 0.034</td>
<td>4%</td>
</tr>
</tbody>
</table>
averaged results are shown in Figures 3-4. For specimens without sodium azide, Figure 3 illustrates that the specimens with the highest concentration of $H_2O_2$ solution added (3%) produced the highest MDA release. The 3% $H_2O_2$ solution was required because the erythrocyte catalase destroys $H_2O_2$ quickly. This destruction of $H_2O_2$ was so rapid that only a small amount of MDA formed. On the other hand, for specimens in which catalase was inhibited by sodium azide, a 0.75% $H_2O_2$ concentration produced the highest MDA release (Figure 4). In addition, there was a remarkable decrease in MDA release at $H_2O_2$ concentrations of 1.5% and 3.0%. This decrease in MDA was most likely due to oxidation of malondialdehyde by the higher concentrations of $H_2O_2$. The optimum $H_2O_2$ concentrations determined both with and without azide agreed with the results of Cynamon et al (90) for a healthy subject. We were unable to perform similar studies with a vitamin E deficient subject because of the small volume of blood we received. However, Cynamon et al found that the optimum hydrogen peroxide concentrations for vitamin E deficient subjects were the same as what was determined in healthy subjects (90).

Anticoagulant Studies

Cynamon et al used only citrated specimens in their
FIGURE 3
Optimum Hydrogen Peroxide Concentration (without azide)
FIGURE 4
Optimum Hydrogen Peroxide Concentration (with azide)
study (90). However, since this anticoagulant is not commonly used in the clinical laboratory, the use of heparin or EDTA was also investigated. A comparison of % MDA using different anticoagulants is shown in Table 2. First, citrate and heparin samples obtained from three healthy subjects were run in parallel. For each subject, the replicate assays for each anticoagulant were averaged and the % MDA calculated. The differences in % MDA between the citrate and heparin samples for the three subjects were 1.4, 0, and 0.2 % MDA, respectively. These differences are negligible. Then, heparin and EDTA samples obtained from two healthy subjects were run in parallel. Similar differences were also observed for these sets. The average difference in % MDA between two different anticoagulants was 0.5%. Thus, the use of heparin, EDTA, or citrate samples was found to be acceptable. Since the red blood cells are routinely washed before the MDA assay, these findings are not unexpected.

Stability Studies

Cynamon et al (90) assayed their samples immediately after specimen collection. However, this practice is often difficult in the clinical laboratory setting. The results of stability studies are shown in Table 3. Specimens were collected in either heparin or EDTA and assayed immediately
## Table 2

### ANTICOAGULANT STUDIES

<table>
<thead>
<tr>
<th>RUN #</th>
<th>ANTICOAGULANT</th>
<th>REPLICATES</th>
<th>% MDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CITRATE</td>
<td>2</td>
<td>0.0</td>
</tr>
<tr>
<td>1</td>
<td>HEPARIN</td>
<td>2</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>CITRATE</td>
<td>7</td>
<td>4.6</td>
</tr>
<tr>
<td>2</td>
<td>HEPARIN</td>
<td>6</td>
<td>6.0</td>
</tr>
<tr>
<td>3</td>
<td>CITRATE</td>
<td>2</td>
<td>3.4</td>
</tr>
<tr>
<td>3</td>
<td>HEPARIN</td>
<td>2</td>
<td>3.2</td>
</tr>
<tr>
<td>4</td>
<td>HEPARIN</td>
<td>3</td>
<td>0.0</td>
</tr>
<tr>
<td>4</td>
<td>EDTA</td>
<td>3</td>
<td>0.0</td>
</tr>
<tr>
<td>5</td>
<td>HEPARIN</td>
<td>8</td>
<td>2.1</td>
</tr>
<tr>
<td>5</td>
<td>EDTA</td>
<td>8</td>
<td>1.1</td>
</tr>
</tbody>
</table>
### Table 3

#### STABILITY STUDIES

<table>
<thead>
<tr>
<th>SPECIMEN</th>
<th>0 HR</th>
<th>24 HR</th>
<th>48 HR</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>4.7</td>
<td>3.5</td>
<td>3.4</td>
</tr>
<tr>
<td>EDTA</td>
<td>5.2</td>
<td>1.6</td>
<td>3.8</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.9</td>
<td>2.1</td>
<td>0.6</td>
</tr>
<tr>
<td>HEPARIN</td>
<td>0.7</td>
<td>1.9</td>
<td>2.3</td>
</tr>
<tr>
<td>HEPARIN</td>
<td>2.8</td>
<td>4.1</td>
<td>5.9</td>
</tr>
</tbody>
</table>
(0 hour), at 24 hours, and at 48 hours after collection. Specimens not assayed immediately were centrifuged, the plasma was removed, and the red cells were stored at 4°C. There was little difference between % MDA results at 0 hr, 24 hr, and 48 hr storage and no pattern of change was observed. None of the differences on these samples would have clinical significance since the upper limit of the reference range was 6% MDA. Most of the differences can probably be attributed to the precision of the method, most notably the very low absorbance readings for specimens without azide. From these studies, I concluded that specimens could be assayed up to 48 hours after collection when the red cells were separated and stored at 4°C. Nevertheless, additional studies on samples from patients with elevated MDA release should be carried out to assay the stability in this result range.

**Precision Studies**

For within-run precision studies, specimens were obtained in both heparin and EDTA from a healthy patient. Each sample was assayed eight times. The results are presented in Table 4. The precision for specimens assayed without sodium azide (standard deviation, 4.0 and 5.4 nmol MDA/mL RBC in EDTA and heparin, respectively) was relatively poor because of the very low absorbance readings
Table 4

PRECISION STUDIES
(nmol MDA/mL RBC)

<table>
<thead>
<tr>
<th>SPECIMEN</th>
<th>REPLICATES</th>
<th>- AZIDE $\bar{x} \pm SD$</th>
<th>+ AZIDE $\bar{x} \pm SD$</th>
<th>CV (+ AZIDE*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPARIN</td>
<td>8</td>
<td>6 $\pm$ 5.4</td>
<td>288 $\pm$ 12.2</td>
<td>4.2%</td>
</tr>
<tr>
<td>EDTA</td>
<td>8</td>
<td>3 $\pm$ 4.0</td>
<td>276 $\pm$ 9.9</td>
<td>3.6%</td>
</tr>
</tbody>
</table>

*CV for samples without azide not calculated.
of these specimens i.e., typically between 0.000 and 0.030. The coefficients of variation for samples assayed without azide are not shown in Table 4. Because of the low readings observed in the absence of azide, the coefficients of variation are extremely high and do not have much meaning. However, the precision for specimens assayed with sodium azide (standard deviation, 9.9 and 12.2 nmol MDA/mL RBC in EDTA and heparin, respectively) was much better. The coefficients of variation (CV) for heparin and EDTA were 4.2% and 3.6%, respectively. The precision of my assays with sodium azide was better than the 4.8% CV for a healthy patient found by Cynamon et al (90). Because of the low absorbance readings of assays without azide, specimens were routinely assayed in duplicate, absorbance readings averaged, and % MDA calculated from the average.

Clinical Studies

Vitamin E status was studied in three groups of patients. The first was the control group which consisted of fasting healthy adult males and females. The second group were male and female children from the Children’s Hospital and Medical Center (CHMC) in Seattle. All of the children except one had cystic fibrosis. The other child had intrahepatic bile duct hypoplasia. Most of the CHMC samples were non-fasting because of the logistics of
specimen collection. The final group consisted of fasting males who were classified as being hyperlipidemic (HLM) on the basis of low density lipoprotein (LDL) cholesterol levels greater than the 75th percentile of the reference population for their age (approximately 150 mg/dL).

The MDA concentrations in specimens assayed without azide for all of the specimens from healthy subjects were in the range of 0-16 nmol MDA/mL RBC while the concentrations for the vitamin E deficient patients ranged from 113-152 nmol MDA/mL RBC. As mentioned previously (see Chapter 1 Introduction), the concentrations of MDA formed in the absence of sodium azide reflect the antioxidant protection of the erythrocyte membranes. Consequently, vitamin E deficient patients exhibit much higher MDA concentrations than healthy patients. Conversely, MDA concentrations determined with azide present were similar for both healthy and deficient subjects and ranged from 144-296 nmol MDA/mL RBC. MDA concentrations in the presence of azide reflect the maximal possible release of MDA due to membrane lipid peroxidation.

Cynamon et al (90) provided a more sensitive assay by calculating the % MDA from the MDA concentration without azide divided by the MDA concentration with azide. This number was then multiplied by 100 to yield the % MDA (fractional MDA release). Cynamon et al observed that patients with plasma vitamin E levels in the reference
range (3-17 mg/L) had essentially no fractional MDA release (% MDA < 5) while patients with plasma vitamin E levels < 2.5 mg/L possessed MDA release values in the 50% range. There were also three patients who had plasma vitamin E levels between 2 and 5 mg/L and MDA release values between 10 and 17 %. For our study, 0-6% MDA was defined as the reference range. This range is in close agreement with Cynamon et al (90) and also reflects the results from our control group. We classified patients with > 50% MDA values as being vitamin E deficient (in agreement with Cynamon) and patients with MDA values > 6% but < 50% as being marginally deficient for vitamin E.

Table 5 shows the mean (±) standard deviation (SD) for plasma vitamin E, % MDA, and vitamin E/total lipids ratio (E/TL) for each of the previously mentioned clinical groups. For this table, the CHMC group is subdivided into three groups. The first group (1CHMC) is classified as the true deficient group with all parameters listed above being in the deficient range. Samples from subjects in the second group (2CHMC) had both plasma vitamin E levels and E/TL levels in the reference range but % MDA values that were elevated. The possible explanations for these discrepancies will be discussed in the text relating to Figure 7. The third group (3CHMC) had assays in the reference range except for a few samples with marginally deficient % MDA results.
### Table 5

#### GROUP COMPARISONS

<table>
<thead>
<tr>
<th>GROUP</th>
<th>(n)</th>
<th>MDA (&lt; 6%)</th>
<th>VITAMIN E* (5-20 mg/L*)</th>
<th>E/TL (&gt; 0.8 mg/g**)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>(27)</td>
<td>2.2 ± 2.2</td>
<td>11.1 ± 2.6</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td>1CHMC</td>
<td>(2)</td>
<td>72.5 ± 19.7</td>
<td>2.4 ± 0.0</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>2CHMC</td>
<td>(2)</td>
<td>65.7 ± 8.5</td>
<td>7.4 ± 0.3</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>3CHMC</td>
<td>(14)</td>
<td>9.7 ± 7.0</td>
<td>8.3 ± 4.2</td>
<td>2.4 ± 1.0</td>
</tr>
<tr>
<td>HLM</td>
<td>(34)</td>
<td>7.3 ± 5.7</td>
<td>14.7 ± 4.1</td>
<td>2.3 ± 0.5</td>
</tr>
</tbody>
</table>

#### KEY:

- **CONTROL** = vitamin E sufficient adult group
- 1CHMC = Children's Hospital and Medical Center
- 1CHMC = true vitamin E deficient group
- 2CHMC = probable vitamin E deficient group
- 3CHMC = vitamin E sufficient group
- HLM = Hyperlipidemic male group
- E/TL = Vitamin E/total lipids ratio

Values are Mean ± SD.

*children's reference range = 3-17 mg/L

**children's reference range = > 0.6 mg/g
The data in Table 5 clearly show a separation of the ICHMC group from the control group for all test categories (% MDA, plasma vitamin E level, and E/TL ratio). There was approximately a thirty-fold increase in the % MDA for the ICHMC group when compared to the control group (72.5 ± 19.7% MDA vs. 2.2 ± 2.2% MDA). Since the size of the ICHMC group was only 2, statistical analysis was not possible and these data should be considered tentative until a larger sample of subjects can be analyzed. However, the values for both groups were similar to the study by Cynamon et al (90) who found a 44.1 ± 18.8% MDA for the vitamin E deficient group and a 2.1 ± 1.4% MDA for the vitamin E sufficient adult group (p < 0.001). Cynamon et al classified their subjects as being vitamin E deficient or vitamin E sufficient by utilizing the conventional methods of assessing vitamin E including plasma vitamin E and E/TL ratio (90).

For plasma vitamin E levels, the ICHMC group and the control group were also very different. The reference range for plasma vitamin E is approximately 3-17 mg/L in children (22) and 5-20 mg/L in adults (97); the ICHMC group had a vitamin E level of 2.4 ± 0 mg L and the control group was 11.1 ± 2.6 mg L. These plasma vitamin E levels were similar to the results of Cynamon et al (90), who observed 1.2 ± 1.9 mg/L for the vitamin E deficient group and 12.8 ±
4.2 mg/L for the vitamin E sufficient adult group \( (p < 0.001) \).

Finally, the E/TL ratios between the ICHMC and the control group were also different. The E/TL ratio is > 0.8 mg/g in reference healthy adults (21) and > 0.6 mg/g in reference healthy children(22). For the ICHMC group the E/TL ratio was 0.4 + .01 mg/g. The E/TL ratio for my control group was 2.3 + 0.6 mg/g. Once again, similar results were observed by Cynamon et al (90). They found that the E/TL ratio for the vitamin E deficient group was 0.2 + 0.2 mg/g and for the vitamin E sufficient adult group was 2.4 + 0.4 mg/g \( (p < 0.001) \).

Figure 5 illustrates the % MDA compared to the plasma vitamin E concentration for the three major groups studied. When 6% MDA is used as the upper limit of the reference range, almost all of the subjects in the control group fall within the reference range. However, some of the HLM subjects and CHMC subjects were also in the reference range. The ICHMC subjects stood apart from all but two of the remaining subjects in their striking \( (> 50\% \text{ MDA}) \) elevation of % MDA. There was also a noticeable increase in % MDA around the 12-14 mg/L plasma vitamin E level for the HLM group. This will be discussed in more detail with Figure 8.

Figures 6-8 show each group from Figure 5 individually. The 27 subjects in the control group, as
FIGURE 5
% MDA vs. Vitamin E

- CHMC
- HLM
- Control
- 6% MDA
shown in Figure 6, all had % MDA values < 6% except for one individual at 7.9% MDA. Each individual of the control group had plasma vitamin E levels between 5 and 17 mg/L which were within the reference range.

The results of the CHMC group are shown in Figure 7. As noted above, the two patients with plasma vitamin E levels of 2.4 mg/L had MDA values > 50%. There were also two other individuals who had MDA values > 50% but their plasma vitamin E levels were at the low end of the reference range (7.2 and 7.6 mg/L). Interestingly, these latter two children are brother and sister. The best explanation for the conflicting results may be found in the children's diet. Their father is a fisherman and their diet is high in polyunsaturated fatty acids, e.g., fish oils. It is well established that an increased intake of polyunsaturated fatty acids results in an increased daily requirement for vitamin E (5,9). This increased requirement for vitamin E, along with the impaired absorption of vitamin E associated with cystic fibrosis, is a plausible explanation for the increased % MDA in spite of reference range plasma vitamin E concentrations observed in these children.

There were also eight patients in the cystic fibrosis group with marginally deficient MDA results ranging from 7.9% to 22% MDA. These children's plasma vitamin E levels ranged from 3.2 to 11.2 mg/L. Whether the results above
FIGURE 6
% MDA vs. Vitamin E (CONTROL)
FIGURE 7
% MDA vs. Vitamin E (CHMC)
6% MDA in these subjects indicates a true deficiency requires additional investigation. Finally, there were two children with 1% MDA values and plasma vitamin E levels between 15 and 17 mg/L. These values for plasma vitamin E are at the upper end of the reference range. It was learned that these children were on vitamin E supplemented diets.

Figure 8 illustrates the % MDA and plasma vitamin E results for the HLM group. The two individuals with an increased plasma vitamin E level (> 20 mg/L), as expected, showed % MDA values in the reference range (0 and 6%). An interesting observation is that approximately one-half of the HLM patients had MDA results greater than 6% (marginally deficient). In fact, the mean % MDA (7.3%) for the HLM group was significantly different from the mean % MDA (2.2%) for the control group (p < 0.001). In addition, the mean plasma vitamin E level (14.7 ± 4.1 mg/L) for the HLM group was significantly different (p < 0.001) from the control group (11.1 ± 2.6 mg/L). These results agree with other investigators and suggest that the increased plasma lipids in these individuals may cause the partitioning of vitamin E off the red cell membrane and into the lipid fraction of plasma (15,78,84). If true, the red cell membrane would have decreased tocopherol and therefore, not be fully protected from oxidation. This loss of protection would be manifested in a moderate increase in % MDA. The
FIGURE 8
% MDA vs. Vitamin E (HLM)
red cell tocopherol that is repartitioned to the plasma would produce a slight increase in plasma tocopherol. The RBC tocopherol concentrations in healthy and hyperlipidemic rats have been shown to reflect tissue concentrations (82,83). Comparisons of RBC tocopherol and tissue tocopherol have not been made in hyperlipidemic human beings; however, Farrell (22) has observed that decreased RBC tocopherol was paralleled by decreased tocopherol in tissues from cystic fibrosis patients. RBC tocopherol concentration was also found to be closely correlated to the platelet tocopherol and E/TL ratio by Mino et al and Kitagawa et al (73,74). From another perspective, Haddad et al placed hyperlipidemic males on a short-term low fat diet and observed a decrease in the plasma vitamin E concentration along with the decrease in lipids. He also observed an increase in the RBC tocopherol concentration (84). Therefore, when the partitioning effect of hyperlipidemia does occur, the % MDA gives a more accurate assessment of biologically active vitamin E than does the plasma vitamin E level.

The association of increased plasma lipids and plasma vitamin E was examined in more detail. In Figure 9, a comparison of plasma total lipids with plasma vitamin E levels for the control and HLM groups is shown. The CHMC group was not included here because most of their samples were non-fasting. As expected, the mean plasma total
FIGURE 9
Vitamin E vs. Total Lipids

△ HLM
■ Control
lips for the control group was lower (493 ± 114 mg/dL vs. 634 ± 112 mg/dL) than the mean for the HLM group (p < 0.001). The correlation coefficient (r = 0.70) showed a positive correlation between plasma total lipid and vitamin E concentrations and is in agreement with other studies (4,15,21).

In contrast, Figure 10 reveals a poor correlation (r = 0.20) when comparing plasma total lipids with % MDA. However, this is not surprising since the comparison is being made between two different type of assays (a static assay versus a functional assay). In this situation, i.e., elevated plasma total lipids, the available RBC tocopherol is actually sequestered away into the plasma lipid fraction so that the plasma vitamin E concentration now reflects both the intake of vitamin E and the sequestration effect. On the other hand, the % MDA appears to reflect the RBC tocopherol concentration and presumably, the tissue tocopherol concentration without any affect from plasma total lipid levels.

Since cholesterol-containing lipoproteins are the major carriers of vitamin E, it was worthwhile to compare cholesterol and vitamin E concentrations. This comparison is shown in Figure 11. The mean cholesterol concentrations of the HLM group (250 ± 34 mg/dL) as compared to the mean values of the control group (195 ± 32 mg/dL) were significantly different ( p < 0.001). The results
FIGURE 10
% MDA vs. Total Lipids

△ HLM
■ Control
FIGURE 11
Vitamin E vs. Cholesterol

- ▲ HLM
- ■ Control
FIGURE 12
% MDA vs. Cholesterol

- ▲ HLM
- ■ Control
indicated that there was also a positive correlation ($r = 0.58$) between cholesterol and plasma vitamin E concentrations but the correlation was not as strong as the correlation between total plasma lipid and vitamin E concentrations.

Finally, Figure 12 shows the cholesterol results compared to the MDA for both groups. Once again, the correlation was poor ($r = 0.28$) but was somewhat better than the correlation between plasma total lipids and MDA. The same reasoning mentioned previously applies as with the plasma total lipid measurement.
In this research, the MDA procedure by Cynamon et al. has been evaluated and refined. In addition to

Cynamon et al. have shown that heparin and EDTA are also

acceptable anticoagulants. Instead of having to assay the

MDA immediately as had been previously reported by Cynamon

elevated the red blood cells at 4°C for up to 48

times after collection also produced accurate results. The

MDA assay does not require specialized equipment and

requires a limited amount of technologist time. The

average amount of time required is approximately 1 mL

of blood. Thus, the MDA has great potential as a

simple, relatively simple, clinical laboratory procedure

designed to measure thiocyanate activity.

The clinical usefulness of the MDA assay

and application to the health care of patients by comparison of

the results obtained with the standard assessment of vitamin E

status in patients are discussed.
Finally, the use of the plasma total lipids in assessing vitamin E status is important and cannot be underestimated. Plasma vitamin E levels are greatly affected by the total lipid levels, whereas, % MDA values appear to be independent of plasma lipid levels and presumably measure functional vitamin E status. Therefore, the % MDA would seem to be a better indicator of vitamin E status than plasma vitamin E levels.

**Further Prospective Studies**

One useful study would involve comparison of the RBC tocopherol concentration to the % MDA, plasma tocopherol, and the E/TL ratio. This would be of special interest in the hyperlipidemic group. The expected result would be a decrease in RBC tocopherol concentration as the plasma total lipid concentration increased because of the partitioning effect mentioned earlier. Results of such a study may be useful in providing an explanation for the slightly elevated % MDA in some hyperlipidemics.

Another possible study would be the confirmation of patients with true vitamin E deficiencies by a different method. In such a study, patients who have increased MDA and decreased plasma vitamin E concentrations would be supplemented with vitamin E for a period of time. Subsequently, assays for plasma vitamin E concentration and
% MDA would be repeated. One would expect that if the patient were truly deficient, both the plasma vitamin E concentration and % MDA would return to the reference range. This approach of supplementing patients could also be used with those patients who had marginally deficient % MDA values (> 6% MDA and < 50% MDA).

The reference range of < 6% should also be further investigated. This would involve collecting more clinical samples from healthy patients, cystic fibrosis patients, and hyperlipidemic patients. Other populations of interest include premature infants and hemodialysis and diabetes mellitus patients. Premature infants commonly suffer from vitamin E responsive hemolytic anemia. Uremic patients on chronic hemodialysis have been reported to have low vitamin E concentrations. Patients with diabetes mellitus frequently have increased lipids. This may cause vitamin E to partition from the red blood cell (and other tissues?) into the plasma as was probably observed in my study. Results from the two previously mentioned proposals concerning the measurement of RBC tocopherol concentrations and the confirmation of true vitamin E deficiencies would also help support this study of the reference range. Interpret a 15% MDA may also involve consideration of the subject's polyunsaturated fatty acid intake and tissue vitamin E concentration from a tissue biopsy.

Finally, a study concerning the effects of lipids
vitamin E status could be done. This would involve increasing dietary lipids for a short period of time in healthy subjects and measuring the plasma vitamin E, E/TL, and % MDA before and during the diet. The same measurements could be done with healthy subjects who were placed on a short-term decreased lipid intake. The results of all of these proposed studies would be useful in determining the assay which is the best indicator of vitamin E status.


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