CO-ORDINATED RESEARCH PROGRAMME ON
DEVELOPMENT AND APPLICATION OF ISOTOPIC TECHNIQUES
IN STUDIES OF VITAMIN A NUTRITION

Report on the First Research Co-ordination Meeting (RCM)
San Francisco, CA, USA
14 - 18 December 1995

INTERNATIONAL ATOMIC ENERGY AGENCY
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A report prepared by the IAEA’s
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FOREWORD

In Vitamin A nutrition, evaluations to ascertain the efficacy of intervention strategies are becoming increasingly important. However, state-of-the-art methods for evaluating vitamin A status often do not provide enough quantitative information on vitamin A status and the bioconversion of carotenoids, particularly in people with subclinical vitamin A deficiency. These limitations have had programmatic consequences. The principal reason the new Coordinated Research programme (CRP) was formulated was to improve techniques for measuring vitamin A status and the bioconversion of carotenoids to vitamin A with the expectation that the new methods could contribute meaningfully to field-based evaluations of the efficacy of intervention strategies. The International Atomic Energy Agency (IAEA) is sponsoring programmes to develop and transfer isotopic techniques to improve nutrition monitoring in developing countries. The New CRP "Development and Application of Isotopic Techniques in Studies of Vitamin A Nutrition" has seven teams, six of which are working to develop methods based on orally administered isotopically labelled retinol which will be a valid measure of whole body retinol (mostly hepatic reserves) and useful under typical field conditions, particularly in women and children with marginal vitamin A deficiency. The seventh team is biosynthesizing uniformly deuterated β-carotene by growing foods in deuterated water. This report summarizes the research to be undertaken, as presented at the first Research Co-ordination Meeting (RCM).

The CRP has benefitted substantially from financial support from the Department of State within the Government of the United States of America. In addition, the first Research Coordination Meeting, the subject of this report, was generously hosted by Dr. Janet C. King and her team at the United States Department of Agriculture, Agricultural Research Service, Western Human Nutrition Research Center, San Francisco, California.

It is also important to note the substantial contributions of the expert consultants who participated in this meeting or who have assisted through correspondence.

On behalf of all the participants in this Coordinated Research Programme, the International Atomic Energy Agency gratefully acknowledges all this support.
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PART I: SUMMARY REPORT
1. INTRODUCTION

Vitamin A deficiency (VAD) continues to impinge on the life-expectancy and well-being of millions of people, many of whom are young children living in Asia, Africa, Eastern Europe, and Latin America. Despite the expenditure of millions of dollars to treat and prevent VAD, it continues to imperil human health and productivity.

Adequate vitamin A can be derived from fruits and vegetables to reduce the prevalence of night blindness, one of the most common signs of Vitamin A deficiency, but the reliability of affordable food sources of provitamin A carotenoids (the precursors of vitamin A) in repleting vitamin A stores in marginally deficient people is debated. This uncertainty is attributed partially to intervention design and evaluation methods, particularly to methodological limitations for classification of marginal vitamin A status, and for reliably measuring dietary intake of provitamin A carotenoids. Better techniques for quantifying vitamin A stores, provitamin A carotenoid intake, and for assessing tissue distribution of vitamin A would provide a much stronger database for monitoring and targeting interventions.

Indicators available for field-based assessments of vitamin A status include the Relative Dose Response (RDR) and Modified Relative Dose Response (MRDR). These tests identify people with low hepatic stores of vitamin A, are indirect but practical and probably closer to measures of hepatic concentrations than is plasma retinol concentration. What is lacking is a reference against which to evaluate the RDR and MRDR. Comparisons of RDR or MRDR results against measured body stores of vitamin A could potentially increase the sensitivity in interpretation of these practical indicators.

Moreover, physiological conditions such as lipid malabsorption, micelle formation, mucosal cell degeneration, general malnutrition, nutritional status vis-a-vis related nutrients such as zinc or protein, dietary fat intake, intestinal parasites, or concurrent infection, may blunt potential improvements in vitamin A status which a ‘good’ dietary intervention could produce. However, the relative impact of such physiological factors is difficult to assess using available indicators of vitamin A status, making it impossible to know with certainty whether a dietary intervention failure occurred as a direct result of the diet’s composition, or whether the failure occurred secondarily to physiological factors which impaired carotenoid absorption or bioconversion or otherwise affected vitamin A status.

The IAEA’s rationale for bringing isotopic techniques into the arena of vitamin A nutrition stems mainly from two facts. First, there is an urgent and important need to quantify improvements in vitamin A nutrition in most regions of the world particularly in people with marginal vitamin A status, and improved biomarkers would help achieve this goal. Second, kinetic models developed from radioactive tracer measurements of vitamin A status have advanced to the point that it makes sense to try to adapt the models to stable isotopic tracers.
The IAEA therefore held a Consultants’ Meeting\(^1\) and funds were obtained to support a CRP from the IAEA and through extra-budgetary sources. Announcement of the CRP was made in mid-1995, seven Research Contracts and five Technical Contracts were funded and six Research Agreements (expert consultants) were awarded.

The first Research Coordination Meeting was held at the USDA/ARS Western Human Nutrition Research Center in San Francisco, California, from 14 to 18 December 1995. Seven Research Contract holders, 7 Research Agreement holders, 5 Consultants, and 3 observers participated and are listed in Part IV.

The following report summarizes the purposes and achievements of the RCM and provides brief summaries of the research within the CRP which was foreseen or underway at the time of the RCM.

2. CURRENT STATUS

2.1. Orientation of research within the CRP

The Agency initiated the CRP because of real and potential advances in isotopic techniques, the demand for more reliable quantitative information on vitamin A stores for use within vitamin A programmes, recommendations regarding roles of isotopic techniques which were made during the consultants’ meeting, and discussions with the various contract holders during the formative stages of their proposals.

Although the isotopic methods have universal relevance, the CRP is devoted to developing and validating methods to serve the needs of developing countries. All of the projects in the CRP incorporate stable isotope techniques into intervention evaluation designs in the field in a developing country. Six of the seven are working to develop methods based on orally administered isotopically labelled retinol which will be a valid measure of whole body retinol (mostly hepatic reserves) and useful under typical field conditions, particularly in women and children with marginal vitamin A deficiency. In the seventh project, uniformly labelled β-carotene is being biosynthesized by growing foods in isotopically labelled water.

In this CRP, each Research Contract (all awarded to developing country experts) is ‘twinned’ with a laboratory in a developed country to which the IAEA awarded either a Technical Contact or a Research Agreement.

2.2. Purposes of the RCM

- Present and debate the hypothesis and design of each study (i.e., each Research Contract)
- Discuss assumptions of the equilibration method and the total traced mass method for measuring hepatic vitamin A, in general, and the relevance to each study design (see Green’s paper in Part II)
- Discuss study design implications using d4-retinyl, d8-retinyl, or both tracers of retinol
- Discuss mathematical models for calculating retinol pool size from D/H ratios and implications of the models for post-dose timing of blood sampling
- Discuss sample size determinations

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• Decide how subjects should be characterized in terms of immune status, nutritional status, physiological condition, etc., that have a bearing on assessed vitamin A status.
• Discuss selected key aspects of carotenoid metabolism (see Southon’s paper in Part II).

3. STUDY TOPIC SUMMARIES

Distribution of the full descriptions of the projects is restricted to participants in the CRP until such time as the studies are either complete and accepted for publication or, otherwise, until the authors and the IAEA agree to release full details.

3.1. Impact on vitamin A status from increasing dietary intake of provitamin A carotenoid-rich foods

Three groups (Thailand, China, Philippines) will evaluate the extent to which specific foods, conventionally thought to be rich in provitamin-A carotenoids, reverse marginal vitamin A deficiency and increase hepatic stores of retinol.

3.1.1. Dietary intake of provitamin-A carotenoids to improve maternal vitamin A stores during lactation (Insitute of Nutrition, Mahidol University, Thailand; Johns Hopkins University, USA).

Maternal vitamin A stores must be adequate to protect the mother, as well as to maintain adequate vitamin A status of breastfeeding infants. Maternal vitamin A deficiency is a pressing public health problem in Thailand. What is uncertain is the extent to which fruit and vegetable sources of pro-vitamin A carotenoids can improve vitamin A status in vitamin A deficient lactating mothers. The project will evaluate the impact of vitamin A intervention in the form of pro-vitamin A carotenoid-rich plant foods on total body stores of vitamin A as measured by an isotope dilution method in lactating women with marginal vitamin A stores. An isotope dilution method will be used to measure vitamin A stores in the women before and after the dietary intervention. The isotopic data will be related to vitamin A status as assessed by conventional indicators in an effort to improve the precision of these indicators, as well as to breast milk vitamin A concentration to determine whether breast milk vitamin A concentration is a reliable predictor of maternal vitamin A status. The project considers the fact that the mammary gland concentrates retinol during the first month of breastfeeding and so focuses on relating breast-milk retinol concentrations to maternal stores later in breastfeeding, i.e. at least 4-5 weeks postpartum. It also considers the importance of relating breast-milk retinol concentration to fat in breast-milk.

3.1.2. Dietary intake of provitamin-A carotenoids to improve preschool children’s vitamin A status (Institute of Nutrition and Food Hygiene, Chinese Academy of Preventive Medicine, China; USDA/ARS Centre in Boston).

Vitamin A deficiency is an important public health problem in China, mainly affecting young children and pregnant women. The Chinese dietary sources of vitamin A are mostly vegetables and fruits but the comparative bioavailability of pro-vitamin A carotenoids from different sources in China is not known. The study will 1) identify pre-school age children with low vitamin A determined from serum retinol; 2) measure their vitamin A stores with isotope dilution methods; 3) measure IgA, IgE, IgM in serum to evaluate immune status and zinc content of hair to evaluate zinc status as a basis of evaluating potential interactions between zinc status and vitamin A responsiveness; 3) provide foods rich in pro-vitamin A carotenoids (carrots, spinach) for 3 months; 4) re-measure vitamin A stores to learn which foods produced the largest increases in vitamin A stores.
3.1.3. Dietary intake of provitamin-A carotenoids to improve school-age children's vitamin A status (Nutrition Center of the Philippines; USDA/ARS Center, Boston).

The objectives of the project are to 1) use stable isotope dilution methods to measure vitamin A stores in Filipino school children; 2) correlate the measured stores with vitamin A status determined by conventional indicators (MRDR); 3) use stable isotope dilution to determine whether the daily feeding for 3 months of 5 to 6 servings of fruits and vegetables increases liver stores of vitamin A in school-age children with marginal VAD as established by serum retinol.

3.2. Nutrient supplements of Vitamin A

Two projects are evaluating a form of vitamin A supplementation in children.

3.2.1. Supplementation with a vitamin A-iron fortified edible sponge (The Hebrew University of Jerusalem, Faculty of Agriculture, Institute of Biochemistry, Food Science and Nutrition)

This project aims to improve nutritional status of children in the northern region of Ethiopia. The objective of the proposed project is to improve the nutritional status of poor communities in Ethiopia through nutrient supplement/fortification, with particular reference to vitamin A. The specific aim is to develop an affordable, stable, and efficacious vitamin A-iron fortified "edible sponge" which is well-tolerated by young children. This work is being done in Israel. The project includes establishing the acceptability of the sponge in studies in children in Ethiopia, assessing the bioavailability of the nutrients in the sponge using isotopic methods, and, if successful, transferring the technology to Ethiopia.

3.2.2. Use of deuterated retinol to assess supplementation (Instituto de Investigacion Nutricion, Lima; University of California, Davis)

The purpose of this study is to determine whether the deuterated retinol dilution (DRD) technique can be used to assess changes in total body stores in response to supplementation with different levels of vitamin A in a population of school children. The project will evaluate the impact of vitamin A supplements (400 and 1200 micrograms RE) on total body stores on vitamin A as measured by isotope dilution in Peruvian school-age children and correlate body store data with vitamin A status assessed by conventional methods.

3.3. HIV and vitamin A

One project is focussing on the transmission of the human immunodeficiency virus (HIV) from mother to infant during birth in mothers with vitamin A deficiency (Department of Paediatrics/child Health, University of Natal, South Africa; Pennsylvania State University)

Transmission of human immunodeficiency virus (HIV) from mother to infant (MTI) occurs in 10 to 52% of pregnancies in South Africa. Many factors contribute to the risk of transmission, including maternal vitamin A status. The hypothesis of the study is that MTI transmission of HIV will be reduced in vitamin A deficient women through vitamin A supplementation. This project will use isotope techniques to measure vitamin A stores in pregnant women to determine the range of vitamin A status in which vitamin A supplementation reduces MTI transmission. It will make additional relevant measures such as of HIV transmission rates and vaginal epithelial cell integrity as described in the proposal. It will also relate maternal vitamin A stores to breast-milk vitamin A concentration.
3.4. Development of methods for measuring carotenoid bioavailability (University of Kerala, India, USDA/ARS, Beltsville)

One project is biosynthesizing uniformly labelled beta carotene to use in feeding studies to measure carotenoid bioavailability (University of Kerala, India).

The objectives of the project in India are to 1) design a controlled system for growing Spirulina in deuterated water; 2) feed the labelled Spirulina produced in step 1 to primates which are housed at the facility in Kerala, India, to measure absorption and bioconversion of beta-carotene to vitamin A; 3) transfer the techniques developed in steps 1 and 2 to studies in pregnant women and children in India to measure effects of environmental factors, such as contamination with lead and/or parasitism, on the nutritive value of beta-carotene.

4. METHODS

4.1. Isotopic and related issues

Most of the discussion are either a) reflected in improved study designs; b) summarized in papers included in this report; and/or c) the subject of investigations which grew out of the collaborative CRP. Highlights of key points are listed here:

• D4-retinyl ester should be administered at dose 1 and D8-retinyl ester at dose 2 because some of the 1st dose will be retained in the retinol pool. A second advantage of using a different tracer for the second study is that the first tracer can be measured over more time to characterize the terminal end of the slope from study 1.
• Sample size should be based on estimated pool size, retention of vitamin A, and if a dietary intervention, on the expansion of the retinol pool expected from quantity of β-carotene provided (see Southon’s paper in Part II).
• Analytical error in each lab needs to be established as part of each protocol.
• Time points for blood drawing remain an area of investigation in the context of comparing the equilibration versus total traced mass approaches. Using the ‘isotope dilution model’ the goal is to identify 2 time points (i.e. 7-14 or 10-20, or 14-25 days post-dose) to calculate the retinol pool size from ‘equilibrated’ isotopic enrichment. Using the ‘total traced mass model’ the goal is to use a multiple compartment model and examine the slopes of enrichments over time (see Green’s paper in Part II).
• Blood carotenoid profiles might be an indication of whether β-carotene is being cleaved. Further, the carotenoid profiles of the diet versus plasma may be an indication of β-carotene bioavailability, cleavage (see Southon’s paper in Part II).
• In dietary intervention evaluations, if effects of receiving large quantities of provitamin A carotenoids are to be measured, carotenoid/retinol intake may dilute the tracer. To prevent this, a recommended trial is to stop intervention for 2 days (cross to a control basal diet) before performing the post dietary intervention evaluation.
• A study is recommended to learn whether multiple small dose of tracer (3 doses/day for 2 days) is more reliable.
• In relation to maternal/infant nutrition, an evaluation is needed of how improvements in maternal vitamin A stores influence the composition of the breast-milk, i.e., it is not enough to know how breast-milk retinol relates to maternal stores; what is needed is information on how much is transferred to the infant, how long it stays there, and then what is needed from complementary foods.
• For comparison of conventional versus isotopic indicators of improvements in vitamin A status, the limits of detection of the isotopic technique need to be factored into calculations to determine the duration and sample size for dietary interventions.
which will be evaluated. At the time of the meeting, additional isotopic data in the various populations of interest were needed. The pool size, which would affect retention, would have to be estimated on the basis of body weight and estimated liver weight.

- Isotopic studies evaluating the efficacy of provitamin carotenoids might be strengthened by the addition of a positive control, i.e., administering to a group which is physiologically comparable with the dietary intervention group an amount of retinol as preformed vitamin A which is comparable to that expected from the provitamin A carotenoids--this might use a ‘best and worse case’ estimate of the bioavailability and bioconversion of carotenoids.

4.2. For evaluating the efficacy of interventions (whether using isotopic or conventional indicators) the following are recommended:

- Socio-economic and nutritional status should be shown to be comparable between intervention and control groups.
- Fat intake should be estimated (not by household purchases but by intake) in individuals studied.
- Use of nutritional supplements needs to be documented
- Medical history for at least one week prior to and then throughout the study
- Age, height, weight need to be recorded according to the duration of protocol
- Haemoglobin and serum ferritin measurements

4.3. Sample size estimation

The sample size calculation should include at least the following:

- an estimate of the increase in total retinol which is theoretically achievable under the treatment conditions
- an estimate of the error of the calculated increase in liver retinol (µmoles/g liver)
- liver retention of retinol
- bioavailability and bioconversion of the provitamin A carotenoids.

Many factors affect the sample size needed to evaluate the effect of a dietary intervention to improve vitamin A status. The following examples illustrate appropriate steps for projects in the CRP. Because it is not possible to accurately predict the amount of beta-carotene absorbed from different fruits and vegetables, the following example illustrates a 2-step approach to estimating the magnitude of increase in whole body retinol which might occur due to i) preformed vitamin A and ii) a red palm oil dietary intervention. An additional estimate would be required in dietary studies to estimate provitamin A carotenoids’ bioavailability.

4.3.1. Theoretical increase in vitamin A due to intake of preformed vitamin A

1. From preformed vitamin A (as a straight-forward example)

If a single dose of 200,000 IU vitamin A (equal to 60,000 µg retinol), is provided, followed by 4-week equilibration period, what is the predicted magnitude of change in terms of µmol retinol/gram liver? (Molecular weight retinol = 286.5 g/mol)

Assumptions:
Liver storage is 50% of the oral dose
Liver weight is 1200 grams
If liver storage is 50% of the oral dose the net increase in whole body retinol is 30,000 µg retinol, or 25 µg retinol/g liver.

The predicted amount of additional retinol which will be delivered during the feeding study as described above is 25µg/286.5 µmol = 0.087 µmol retinol/g liver.

2. Retinol increases due to dietary supplementation with red palm oil

Assumptions:
Liver storage is 50% of the oral dose
Liver weight is 1200 grams

What is the predicted magnitude of change in terms of µmol retinol/gram liver?
(Molecular weight retinol = 286.5 g/mol)

Dietary treatment:
20 grams oil per day for 10 weeks from either red palm oil or stripped oil (placebo)

Provitamin A content of red palm oil is 30 mg/100g oil;
30,000 µg/100g = 300µg/g oil;
300 µg/g oil x 20 grams oil per day = 6000 µg provitamin A/day

6000 µg provitamin A/6 RE = 1000 µg Retinol/day

If liver storage is 50% of oral dose, the net increase in whole body retinol is 500 µg retinol/day.

500 µg retinol/day x 7 days/week x 10 weeks = 35,000 µg retinol delivered to the liver during the feeding study. The predicted amount of additional retinol which will be delivered during the feeding study is 29.17 µg/286.5 µmol = 0.10 µmol retinol/g liver. This figure would then be used in the calculation of sample size below.

Once the theoretical estimate of the increase in retinol is made, a sample size can be calculated, as illustrated below. Once a sample size is estimated, (which may be within time or budget limits), it may be necessary to either increase the 'dose' (provitamin A carotenoid intake, bioavailability, etc.), or the duration of the intervention to achieve more 'net intake'.

Estimates of each factor, and of the total sample size needed to test the efficacy of a vitamin A intervention, are illustrated below.

3. Mean and SD of calculated liver retinol concentration

Although data will soon be available from several sources, including the IAEA’s Coordinated Research Programme, for calculating ‘change’ within subjects, appropriate human data on which to base this calculation are presently unavailable. Therefore, estimates of sample size presented below were calculated on the basis of data from independent subjects.² The mean and SD of calculated increases in liver retinol which correspond to a ‘change’ in measured liver retinol of 0.30 µmoles was calculated (Southon) as a basis for the sample size estimates shown in the box below. The mean calculated change corresponding to a measured change of 0.30 µmoles/g liver is 0.308, SD 0.182. The

sample size was calculated from the ‘standardized difference’, ie., the ratio of the mean calculated ‘change’ in liver concentration / SD of the ‘change’, power = 0.8, P<0.05.

<table>
<thead>
<tr>
<th>Increase in liver vit.A (mmole/g liver)</th>
<th>Standardized difference</th>
<th>Total no. of subjects per study, i.e. divide by 2 for 2 groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.1/0.182</td>
<td>100</td>
</tr>
<tr>
<td>0.2</td>
<td>0.2/0.182</td>
<td>26</td>
</tr>
<tr>
<td>0.3</td>
<td>0.3/0.182</td>
<td>12</td>
</tr>
<tr>
<td>0.4</td>
<td>0.4/0.182</td>
<td>8</td>
</tr>
<tr>
<td>etc.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The actual studies within the IAEA’s Coordinated Research Programme will evaluate retinol increases within subjects, so that the sample size estimates above based on ‘independent’ subjects would be slight overestimates.

4.4. Additional evaluations to compare isotopic data with conventional indicators of vitamin A status

In addition, the CRP (through one or more projects) is aiming to do the following:

- Compare vitamin A concentration in breast-milk with maternal vitamin A stores measured isotopically to evaluate the comparability of the two methods for determining maternal vitamin A status (South Africa; Thailand)
- Relate isotopic data on vitamin A stores to conventional indicators to improve the precision of the conventional indicators.

A number of other ideas were discussed, and some adopted, with the goal of strengthening the validations or dietary evaluations. Many of the ideas are under evaluation in various studies, and will be discussed in publications from the CRP. One piece of advice is to try matching the study protocol in the Philippines with the one in Thailand taking note that one strength of the Thai protocol is the pre-mixing of the vegetables.

4.5. Publications policy

The Agency strongly encourages and supports the publication of scientific data arising out of these studies. All CRP participants are therefore strongly recommended to publish their data as soon as possible in a reputable peer-reviewed international or national scientific journal. In all cases, the Agency’s support should be acknowledged, and a copy of the manuscript should be sent to the Agency’s technical officer for comments prior to being submitted to the journal. If significant scientific input was obtained through the CRP, then authorship should be discussed among all relevant persons. These issues should be handled in a transparent fashion and coordinated between the first author and the technical officer.

A different policy applies to publications describing the results of projects that have been evaluated centrally. The preparation of such publications will, in the first place, be the responsibility of the Agency’s technical officer. Such publications will be co-authored by all CRP participants who have contributed in any meaningful way to the outcome of the study.

4.6. The next RCM

The next RCM is presently foreseen to take place somewhere in the USA probably in September 1997.
PART II: BACKGROUND PAPERS
1. INTRODUCTION AND SCIENTIFIC BACKGROUND

My purpose here is to discuss the applicability of isotope dilution methods to the assessment of vitamin A status in humans. First, I will briefly review the oral isotope dilution method for assessing vitamin A stores [1,2]. Then I will highlight some aspects of vitamin A intake and metabolism that relate to the oral isotope dilution method. Next I would like to propose some areas of methodological research and development that I feel are needed to advance, validate and improve this technique. Finally, I will make some comments about issues that may require our special attention as we plan stable isotope studies during pregnancy and lactation.

Before reviewing the oral isotope dilution method, I would like to remind us of the concept on which isotope dilution methods are based and to rethink the use of the term "isotope equilibrium." Regarding the original concept, we need to remember that it was developed for a closed system. If, for example, one added a known amount of dye to a beaker of water, then thoroughly mixed the solution and determined the concentration of the dye, one could calculate the volume of water present in the beaker as

\[
\text{volume of water} = \frac{\text{dye added}}{\text{equilibrium dye concentration}}. 
\]

Obviously, in real life we will not be studying a closed system that has reached an isotopic steady state: humans are open systems in a non-equilibrium and typically also a non-steady state. Fortunately, as I will discuss later, meaningful advances have been made in adapting the isotope dilution method to dynamic open systems, but we must always be aware of the assumptions underlying the original equation.

Also, I propose that we stop using the term "equilibrium" (as in "isotope equilibrium") when we are using this type of methodology for in vivo experiments. By definition, an isotope is said to be in equilibrium when the rate of input of tracer equals its rate of output, and the specific activity ratio of tracer/tracee has reached a plateau in the system. Because of the dynamic behavior of vitamin A, such a state does not typically exist with respect to administered tracers for vitamin A, except in special situations resulting from a long period of continual administration of the tracer. In a more usual case, tracer disappearance will follow parallel kinetics (but at different specific activities) in all possible sampling sites, once the vitamin has thoroughly mixed with the exchangeable pools of vitamin A. This fact is often referred to in the tracer kinetics literature as the precursor-product relationship [3].

Now I would like to discuss what we are calling the oral isotope dilution method. I will present the equation published by Furr et al. [2] and describe how the authors intended it to be used to estimate total liver vitamin A in projects such as those we are planning at this workshop. The equation is:

\[
\text{TLR} = F \times \text{dose} \times (s \times a \times [H/D - 1]) 
\]

\(\text{TLR}\) is the total liver reserve of vitamin A (nmol). \(F\) is the fraction of an orally administered dose of deuterated vitamin A that is absorbed and retained in the liver; blood must be sampled long enough after dosing to allow the label to have mixed with
endogenous stores. In previous studies (1,4), blood was collected at 14 or 26 d, respectively. \( F \) is most commonly assumed to be 0.5, and has been shown to range in rats from \( 0.13 \) to > 0.8 at low versus high liver vitamin A levels, and to average 0.49 ± 0.05 (mean ± SD) in sheep [1]. Dose is the mass of deuterated retinol in the administered dose (mmol). The parameter \( s \) is the ratio of the specific activities (D/H) of retinol in serum to liver at the time of blood sampling. Often people incorrectly equate the D/H stable isotope ratio with tracer specific activity (dpm/mass). As pointed out by Cobelli et al. [4], a transformation of the data is required to arrive at an equivalent entity. The value for \( s \) was determined to be 0.65 ± 0.15 in rats [5] and 0.68 ± 0.27 (mean ± SD) in humans [2]. \( a \) is a factor that was added by Furr et al. [2] to account for the disappearance of tracer from the body during the mixing period and was calculated as

\[
a = e^{kt}
\]

where \( k = 1/140 \text{ d} \) (140 days is the estimated half-life of vitamin A turnover in the liver) and \( t \) is the time (d) at which the blood sample is taken following dose administration. Typically, half-life \( (t_{1/2}) \) is converted into a rate constant by using the following equations:

\[
y(t) = y(t_0)e^{kt} \text{ and } 0.5 = 1 • e^{kt_{1/2}}.
\]

Solving for \( k \), one obtains \( k = \ln 2/t_{1/2} \). Sauberlich et al. [6] measured the \( t_{1/2} \) for vitamin A to average 144 days (range, 75 to 241) in 8 adult male volunteers. Therefore, \( k \) would equal 0.0048 day\(^{-1} \) (range, 0.0029 to 0.0092). H/D is the ratio of \( ^1H \) to \( ^2H \) in plasma retinol at the time of sampling. Finally by subtracting 1 in the equation for TLR, one removes the contribution of the deuterated retinol dose from TLR. That is, the equation calculates the pre-dose total liver reserve.

If TLR is being calculated at two times (e.g., before and after a vitamin A supplementation regimen and one discovers in the first test that the dose caused a substantial perturbation of TLR, then \( a \) must be computed from the first to the second determination of TLR. The equation for comparing the first TLR with the second becomes

\[
\text{TLR} = F \ast \text{dose} \ast (s \ast a \ast [H, D + 1]).
\]

Based on the above discussion, it is clear that the coefficients (\( \beta s \)) in the regression equation of Furr et al. (i.e., \( F, s, \) and \( a \)) are not constants but rather unknowns with certain confidence intervals. As I will mention again later, this leads us into an area of multiple regression called by some "constrained multiple regression". As the equation is currently being applied (i.e., using the mean values for these regression coefficients), it will provide more accurate prediction of liver stores for groups of individuals that it will for individuals in a particular group.

2. VITAMIN A METABOLISM AND THE ISOTOPE DILUTION METHOD

Vitamin A metabolism and kinetic behavior have been discussed in detail in several recent reviews [7,8]. Here I will briefly overview these topics and emphasize particular features that will affect results of the studies we are planning. First, we need to consider the effects on vitamin A dynamics of the amount of vitamin that will be administered in order to label the tracee system with enough isotope for accurate, long-term detection. Because of the currently-known analytical detection limits for the
available stable isotopes of vitamin A, investigators will be forced to administer a dose (>3 mg) that will perturb the underlying tracee system (that is, the dose will be large enough to alter kinetics of endogenous vitamin A and body vitamin A stores). To a kineticist, this represents a solvable but definitely more complex situation than administration of tracer amounts of a compound, as could be done with tritiated vitamin A.

One "extra-metabolic" feature that requires serious thought is dietary intake of vitamin A during the field study. We will consider the ramifications of level of intake below, but I note here that it will be very useful to have accurate estimates of intake during the study, since we know that the newest dietary vitamin A entering the liver is the first out into the blood [9,10]. This occurrence will dilute the plasma D:H ratio.

Several characteristics of vitamin A metabolism per se may have an impact on use of the oral isotope dilution method. One consideration is that vitamin A absorption is variable (e.g., definitely among individuals, and possibly as a function of fat intake, vitamin A status and level of vitamin A intake). In studies using lymph-duct cannulated rats [11], we determined that the absorption efficiency for vitamin A averaged 76% ± 16 (mean ± SD, n=11). That is, the coefficient of variation was high (21%). More work needs to be done to develop methods for determining vitamin A absorption efficiency in a given individual, as well as the efficiency of bioconversion of carotenoids to vitamin A.

Fig. 1 presents a simplified overview of vitamin A metabolism; see references 7 and 8 for more details. Briefly, preformed dietary vitamin A or provitamin A carotenoids are absorbed into the enterocytes of the upper small intestine as retinol and carotenoids, respectively. There, a portion of the carotenoids will be converted to retinol. Retinol is esterified, primarily by the enzyme lecithin:retinol acyltransferase at the endoplasmic reticulum, which is the site of production of absorptive lipoproteins (chylomicrons). The retinyl esters will partition into the core of the chylomicron along with cholesteryl esters and triacylglycerols. Nascent chylomicrons are processed through the enterocyte and secreted from the cell by exocytosis. Since chylomicrons are too large to fit through interendothelial spaces or fenestrations in the capillary wall, they enter the lymphatic system and are transported through the thoracic duct to plasma via the subclavian vein in the shoulder. In plasma, chylomicrons acquire apolipoprotein CII from high density lipoproteins and will begin to be metabolized by the enzyme lipoprotein lipase (LpL) which is located on the endothelial surface of capillaries (primarily in adipose tissue, muscle and lactating mammary glands). LpL action accounts for the extrahepatic removal of ~90% of chylomicron triacylglycerols and variable amounts of vitamin A. This uptake of vitamin A may be by monomolecular transfer or particle uptake by extrahepatic tissues. Such an extrahepatic clearance of chylomicron vitamin A may be an important route of delivery of vitamin A to mammary tissue, especially during late pregnancy and lactation. This possibility needs to be considered when using isotope dilution methods in pregnant/lactating women.

The remaining "dilapidated" chylomicrons (chylomicron remnants) are then cleared by the liver parenchymal cells (hepatocytes) via receptor-mediated endocytosis. The retinyl esters are hydrolyzed and the resulting retinol is either bound to apoRBP (retinol-binding protein) in the endoplasmic reticulum for secretion into plasma or transferred to liver perisinusoidal stellate cells for storage as retinyl esters. The fate of retinol in the hepatocytes depends on the load of vitamin A entering the liver: if the dose is large, as will be the case in our studies as currently proposed, some of the newly-arrived retinol will be re-esterified in hepatocytes, waiting its turn for an opportunity to be transferred out on RBP. This delay in export from hepatocytes may be hours to days, depending on the incoming load of vitamin A. Based on studies in
animals, it is likely that, in subjects with more adequate liver stores, more of the dose will be sequestered in stellate cell retinyl ester pools, whereas in the vitamin A-deficient state, most of the dose taken up into hepatocytes as part of chylomicron remnants will be exported directly into plasma. Vitamin A metabolism by the two cell types will be affected by level of dietary intake during the isotope dilution test and will be perturbed by the orally-administered dose of labeled vitamin A.

In stellate cells, stored retinyl esters become a source of plasma retinol \[10\]. Stellate cell retinyl esters undergo a slow but continuous hydrolysis and re-esterification before secretion. It may be the plasma apoRBP to holoRBP (holoRBP is retinol bound to RBP) ratio, and the stellate cell apoCRBP (cellular retinol-binding protein) to holoCRBP ratio, that help determine the timing of mobilization of retinol from stellate cells. An important fact for our proposed studies is that there is a continual turnover of stellate cell retinyl ester stores that results in the mixing of "new" vitamin A with "old." As discussed below, it has also been noted that as stellate cell vitamin A stores get large (i.e., at higher and higher levels of vitamin A status), mixing of "new" and "old" is incomplete. Thus for our purposes, we need to keep in mind that some of the retinyl esters may not mix and exchange in a reasonable amount of time (e.g., <50 days) with the administered stable isotope ("new" vitamin A). This possibility will affect the calculation of isotope dilution, as was observed by Bausch and Rietz in rats \[1\].

Plasma transport, recycling, tissue uptake and utilization of vitamin A are also dependent on vitamin A status. Let's begin with data from animal studies \[12,13\], in which we know not only vitamin A intake but also the liver vitamin A stores, in order to get some insights into the influence of vitamin A status on the kinetic behavior of vitamin A. Rats at one of four levels of vitamin A nutriture (liver levels of \(~8 to ~9700

nmol) received a nonperturbing IV dose of [\(^3\)H]retinol-labeled plasma that had been prepared in donor rats. The disappearance of administered [\(^3\)H]retinol from plasma is shown in Fig. 2. The terminal slope of the curves is proportional to the fraction of the exchangeable pool of vitamin A that is irreversibly utilized per day and is inversely proportional to the liver stores of vitamin A. The figure inset reveals that, initially (before 12 h after dose administration), there is little influence of vitamin A stores on the kinetic behavior of the vitamin. By ~3-4 d after dosing, the maximum divergence, reflecting differences in liver vitamin A stores, is evident. Soon (by 7-10 d), this divergence is lost at lower liver reserves of vitamin A. Let me also point out that we have used early data (3-6 d post dosing) from this type of study to develop prediction equations for estimating liver vitamin A levels based on a single blood sample after both IV \[14\] and oral administration of [\(^3\)H]retinol \[15\].

Another impact of vitamin A status on vitamin A kinetics is illustrated in Fig. 3 which provides information on the recycling of [\(^3\)H]retinol from extravascular pools in rats with high or low liver reserves of vitamin A. Plasma tracer disappearance data from rats with low or high liver vitamin A levels were fit to a 3-compartment model (Fig. 3) which is postulated to represent the central plasma retinol compartment exchanging with two extravascular vitamin A pools (one slowly turning-over and the other fast turning-over). When liver stores are low, the model predicts that there is extensive recycling of the label from the fast turning-over extravascular vitamin A pool and very little dilution of the label in the small, slow turning-over extravascular (storage) pool. Thus the labeled material quickly mixes with body pools of vitamin A and it leaves the system relatively rapidly via degradative pathways. In contrast, in rats with large stores of vitamin A, the portion (~1/3 of the plasma retinol turnover) of the tracer that goes to the slow turning-over pool gets sequestered there and thus retinol entering the plasma from this pool is of low specific activity. As a consequence, the input of tracer into plasma from both extravascular pools is reduced, in comparison to rats with low vitamin A status. For our purposes, it is important to note that, if such an extensive recycling of retinol to plasma
(78 to 13 times before irreversible utilization) did not occur, the effects of vitamin A stores on plasma retinol specific activity might not be seen.

Similar kinetic behavior has been observed in the limited data that are available for vitamin A turnover in humans. Older data collected by the late DeWitt Goodman are shown in Fig. 4. Three adult subjects received an IV dose of \(^{14}C\)retinol; disappearance of label from plasma was monitored for up to 240 d. Unfortunately, no blood samples were collected during the first 18 h after dose administration. Note that the pattern of disappearance is similar to that which we had observed in rats with adequate stores (Fig. 2). The average terminal slope of the tracer disappearance curves was 0.58%/day, indicating that <1% of the body exchangeable pool of vitamin A was irreversibly utilized each day. We [9] used model-based compartmental analysis with the conversational version (CONSAM [16] of the Simulation, Analysis and Modeling computer program (SAAM) [17] to fit these data to a three compartment model (Fig. 5). By simulating the response predicted by this model, we estimate that the fraction of the \(^{14}C\) dose administered peaked in the fast turning-over extravascular pool (compartment 3 in Fig. 5) at 0.6 d; that for the first 5 d, almost all of the input of tracer to plasma was from this pool; that tracer peaked in the slow turning-over extravascular pool (compartment 2, the storage pool) at 10 d (55% of the dose) but most of what was going to get there had arrived by 2-3 d (47% of the dose). These predictions emphasize again the extensive recycling of retinol to plasma from the liver and extrahepatic tissues prior to irreversible loss, as well as the mixing of plasma retinol with liver stores of retinyl esters.

In addition to the data of Goodman, I have more recently had the opportunity to work with the as-yet unpublished work of Doris von Reinersdorff (Hoffmann-La Roche, Nutley, NJ). Since these data are not yet published, I will show an example here at the workshop but will not include data figures in the report. \(^{13}C_3\)-Labeled vitamin A (105,000 nmol [30 mg]) was administered orally to 11 subjects; the appearance and disappearance of retinyl esters, retinol \(^{12}C\) and \(^{13}C\), and retinol metabolites were followed in plasma for 7 d. Currently we are developing a multicompartmental model to interpret these unique and valuable data. Based on results to date, the model predicts that 55% of the dose was absorbed in this example subject. Both the data and the model demonstrate how such a large load of labeled vitamin A perturbs the underlying tracee system. A less dramatic perturbation will occur using doses we plan to administer in the field studies being discussed at this workshop.

In the experiments we are planning at this meeting, we hope to make use of the observation [15] that there is a "window of separation" in the fraction of a dose of vitamin A in plasma between 3 and 6 days after administration of an oral dose of labeled vitamin A to rats (Fig. 6). This separation is due to differences in the liver reserves of vitamin A (Fig. 7). Non-linear regression was applied to the data shown in Fig. 7 in order to develop a prediction equation for liver vitamin A content:

\[
\text{LIV A (nmol)} = 58,577 \exp(-2715 \times \text{FD}_{P3D}) + 1810 \exp(-127 \times \text{FD}_{P3D})
\]

where \(\exp\) is the base e of the natural logarithm, and \(\text{FD}_{P3D}\) is the fraction of the oral dose of \([^{3}H]\)retinol in plasma at 3 d. The Spearman rank correlation \((r_s)\) between observed and predicted liver vitamin A reserves over this large range (5 to 22,000 nmol or 0.48 to 1629 nmol/g) was 0.982. Thus, at 3 d after dosing, at least in rats, there is a sensitive inverse relationship between liver vitamin A levels and the fraction of an oral dose of labeled vitamin A present in plasma. Although the relationship is not linear, the correlation coefficient \((r)\) is 0.509 \((P<0.01)\). I will return to this point later when discussing various possibilities for assessing liver vitamin A stores after an oral dose.
Over the years, one of the main goals of my laboratory's research on the kinetic behavior of vitamin A has been to elucidate the determinants of vitamin A requirements. From an input-output perspective, these determinants relate to food preparation, availability of vitamin A from the diet, absorption efficiency and utilization rate. One of the kinetic parameters we calculate from data collected in in vivo turnover studies is the disposal rate of vitamin A (i.e., the rate at which vitamin A is irreversibly utilized to fulfill vitamin A function and non-vitamin A-related degradative processes). In Fig. 8, I present the relationship between plasma retinol levels and vitamin A disposal rate [13]. These data combine the results from several published and unpublished studies done in my lab with rats at different levels of vitamin A nutriture. Note in Fig. 8 that, as plasma retinol increases, there is an increase in vitamin A utilization. At the present time, we believe that only a small fraction of this can be attributed to vitamin A-related functional utilization, while the majority may be what we call "degradative preservation". This possibility is particularly important for open systems: if a substance is taken in, there had better be a means of disposing of it. Thus, although mammals are quite efficient at storing vitamin A, at least one species among this class (i.e., rats) is able to vary vitamin A degradation rates. In this regard, I appreciate the comment made by Moore [18] in his classic 1957 text: "In interpreting observations on the rate of fall in the reserves of vitamin we may then be able to decide how much of the vitamin was being used for physiological purposes, and how much is 'running to waste'."

Concluding the discussion of determinants of vitamin A utilization, in Fig. 9 is shown data and a multiple regression equation we have developed [13] to predict vitamin A disposal rate in rats (n=62) as a function of plasma retinol, vitamin A intake and liver vitamin A levels. The coefficient of multiple determination ($R^2$) was 0.91 ($p<0.001$). Sixty-eight percent of the variation in disposal rate was predicted by plasma retinol, 18% by liver vitamin A and 14% by intake. These results imply that a pool of retinol which equilibrates with plasma retinol is a site of vitamin A degradation: as plasma retinol increases, so does degradation. When plasma retinol is at its homeostatic upper limit, further increases in vitamin A intake or stores can result in higher rates of vitamin A degradation.

Finally, high environmental temperature, as well as infection and other inflammatory responses, have been shown to lower plasma retinol and RBP levels, and also probably hepatic secretion of retinol. Thus we need to think about how these factors might affect the mobilization into plasma of our oral load of deuterated retinol. This would result in an overestimate in our calculation of total liver vitamin A reserves in the populations we will be studying. We will return to this point in the next section.

3. AREAS OF RESEARCH AND DEVELOPMENT THAT NEED TO BE CONSIDERED

In this section, I present issues related to the isotope dilution method that require future research and/or development. Some of these are important no matter what we eventually choose for an isotope dilution method; others relate directly to the methods of Bausch and Rietz [1], and Furr et al. [2], or to potential modifications of those techniques.

3.1. General issues

First, it will be critical to determine what the ideal stable isotope tracer will be for the proposed field studies. In view of the chemistry of our tracee, we will be considering $^{13}$C versus $^2$H. While the former isotope is more stable in the vitamin A molecule, the natural abundance of $^{13}$C is much higher than that of $^2$H, creating the potential for signal-to-noise problems in our analytical procedures. In addition, $^{13}$C-labeled compounds are more expensive than those labeled with $^2$H. If we choose $^{13}$C, we must ascertain that an
ample supply of uniformly labeled vitamin A will be available. If we choose $^2$H, we need to be vigilant in determining that the preparation is not contaminated with $^3$H (i.e., radioactive tritium). Unfortunately, our choice of tracer may be influenced by factors of economics ($^{13}$C will be significantly more expensive) and availability (presumably, an ample supply of $^2$H will be easier to find). Furthermore, if a second assessment of liver stores is planned, then a different isotope should be used (e.g., if tetradeterated retinol is used for a first test, then the octadeterated form would be suitable for the second assessment).

Second, it will be useful to examine alternative approaches to a single oral dose, with the aim of reducing the acute load of administered labeled material while increasing the isotope enrichment in plasma. Two approaches seem worth investigating: repeated oral doses given within ~24 h and daily oral doses during the "mixing" period.

Third, researchers will need to develop/refine the isotope dilution method for subjects of different ages and in various physiological states. For example, the ideal time for blood sampling is likely not the same under all conditions. Pilot studies should be planned on small numbers of representative subjects for this purpose. Such studies will also allow the analytical teams to provide input on the oral dose required to give adequate detection capabilities at the appropriate sampling time(s).

Fourth, method(s) must be developed to determine the efficiency of vitamin A- and carotenoid absorption, and carotenoid bioconversion, in various populations and from different food sources. Surprisingly, even in animal models, simple and reliable methods for measuring (pro)vitamin A absorption and carotenoid bioconversion have not been worked out, and thus influences of physiological state, age, food source and preparation methods have not been adequately studied.

Also it will be important to measure vitamin A utilization (disposal rate) in different populations and physiological states, since this will not only influence setting requirements but also the value of $a$ in the equation of Furr et al. [2].

Finally as noted above, researchers need to examine effects of inflammation on plasma isotope dilution during the "mixing" period, in view of the findings that vitamin A metabolism is altered in this state such that application of the equation of Furr et al. may overestimate liver reserves of vitamin A [2].

3.2. Topics related to the methods of Bausch and Rietz [1] and Furr et al. [2]

In order to investigate the influence of estimated vitamin A stores on plasma isotope dilution, the isotope dilution test needs to be done first in subjects with low vitamin A status. Then the test should be repeated after increasing the subjects' supplemental vitamin A intake. I believe that Marjorie Haskell and Ken Brown (U.C. Davis) are already doing this type of study. A related research project should be to empirically determine the influence of variable vitamin A intake on the plasma isotope dilution value during the "mixing" period.

Attention to several more theoretical issues would improve the usefulness of the isotope dilution method for assessing vitamin A stores. First, it would be worthwhile to apply more sophisticated multiple regression techniques (such as constrained regression analysis) to defining the values for F, s and a in the equation of Furr et al. [2]. This approach would allow us to assign confidence intervals to these coefficients and to obtain confidence intervals (i.e., a range) for the estimates of stored vitamin A, rather than a single value. For this purpose, we may wish to consult in advance with investigators who have expertise in advanced regression methodologies.
Also, as noted above, one might apply the transformation described by Cobelli et al. [4] to plasma isotope enrichment data in order to account for the presence of pre-dosing plasma tracer and for the amount of tracee in the dose. I would also recommend that the analytical methods be set up so that we can estimate the fraction of an oral dose present in plasma at the time of sampling. This would require the availability of an appropriate internal standard, an estimate of plasma volume and an accurate determination of the amount of label administered. By determining the fraction of dose in plasma, one removes an added source of potential error (i.e., determining tracee in plasma). Further, such an approach may reduce the influence of dietary vitamin A intake during the isotope dilution period on the fraction of administered dose in plasma, since we would be determining the tracer levels in plasma, rather the tracer/tracee ratio (which is more influenced by tracee input from the diet).

3.3. Topics related to modified isotope dilution methods

Several areas of research/development come to mind as one reviews published modifications of the isotope dilution method as originally proposed for assessing vitamin A stores. We may be able to develop several measures of vitamin A stores from the results of a single isotope dilution study. Four methods in addition to the method of Furr et al. [2] come to mind. First, it would be useful to more carefully define isotope kinetics during the "mixing" period. By so doing, it is possible that we might find an earlier, more optimal sampling "window" that reflects liver vitamin A stores. Based on currently-available data from rat and human studies [9,15], I estimate that an ideal time might be at 3-4 d after isotope administration. It would be useful to then empirically derive equations relating vitamin A stores to tracer in plasma at 3-4 d as we have done in the rat [15]. If it is not feasible to collect a sufficient number of liver biopsies for this purpose, we could mathematically relate total traced mass of vitamin A (MT) to tracer in plasma at various times; methods for calculating MT are presented later.

Second, I propose that we apply slope analysis to data collected when the tracer "die-away" curve has reached its terminal slope. Normally an inverse relationship exists between slope (fractional catabolic rate of the system) and vitamin A stores. Determining the slope would also provide the information needed to calculate a in the equation of Furr et al. [2].

Another kinetic approach is available that allows us to determine a subject's total traced mass of vitamin A (MT). It is potentially feasible to apply this technique in a small number of subjects in target populations. I will briefly describe the experimental approach we have used to estimate MT in rats before describing the kinetic methods. I will conclude by mentioning modifications of these procedures that would be needed for application in humans.

We have used rats as an animal model for studying several aspects of vitamin A metabolism/assessment that seem relevant to these factors in humans. Obviously, with rats one has the luxury of controlled conditions with respect to genetics, dietary vitamin A etc., and the possibility for long-term, serial sampling of blood, excreta and tissues. Although such luxuries are never available in human experimentation, I firmly believe that a good animal model is the place for development of methods that can be modified to provide useful information in humans.

In order to measure MT in rats, we administer an IV pulse dose of [3H]retinol-labeled plasma to recipient rats. Labeled plasma is obtained from a vitamin A-deficient donor rat at ~100 min after administration of a dispersion of [3H]retinol in Tween 40. We then sample the plasma of recipient rats for ~40 days, until the plasma [3H] disappearance curve is well into a terminal slope (i.e., on a semilog scale, the
disappearance curve fits a straight line, allowing extrapolation to infinity). At early
times, plasma is sampled frequently so that the shape of the plasma $^3$H disappearance
curve can be accurately defined; later blood samples are more widely spaced. In order to
initially choose sampling times, it is useful to apply a geometric progression. More
details on experimental design and methods have been published [19].

We have used two kinetic approaches to analyze our data on the fraction of
injected isotope dose remaining in plasma versus time: model-based compartmental
analysis and empirical analysis [12,20,21]. For model-based compartmental analysis,
plasma data are fit to a 3- or 4-compartment model using CONSAM [16]. We calculate
the plasma retinol pool size $M(1)$ from the measured plasma retinol concentration and
the estimated plasma volume. Then, assuming a steady state, we use CONSAM to
estimate the mass of vitamin A in other compartments $M(l)$ as well as many other
interesting kinetic parameters, including disposal rate. Finally, the total traced mass $M_T$
is estimated as the sum of the $M(l)$s.

In order to use empirical analysis to estimate $M_T$, data on fraction of dose in
plasma versus time are fit to a 3- or 4-component exponential equation of the type:

$$y(T) = \Sigma I(i) \cdot \text{EXP}[g(i) \cdot T]$$

where $I(i)$ are the intercepts, $g(i)$ are the slopes, EXP is the base e of the natural
logarithm, and $T$ is time. The sum of the intercepts $I(\Sigma i)$ is normalized as follows:

$$H(i) = I(i)/\Sigma I(i).$$

Then one calculates the mean sojourn time (MST) of labeled vitamin A as

$$\text{MST} = \Sigma (H(i)/g(i)^2)/\Sigma (H(i)/g(i)) = \int y(T)^*TdT / \int y(T)dt$$

where $\int$ is the integral from time zero to infinity. The fractional catabolic rate (FCR)$_p$ of
labeled vitamin A as

$$\text{FCR}_p = 1/T_p$$

where $T_p$ is the residence time for the label in plasma and is computed as

$$T_p = \text{AUC}_p = \Sigma H(i)/g(i)$$

where $\text{AUC}_p$ is the area under the plasma tracer response curve integrated from time zero
to infinity. Then vitamin A utilization rate (disposal rate, DR) is computed as

$$\text{DR} = \text{FCR}_p \cdot M_p$$

where $M_p$ is the plasma retinol pool size $[M(1)$ above]. Finally, total traced mass of
vitamin A ($M_T$) is calculated as

$$M_T = \text{DR} \cdot \text{MST}.$$  

An estimate of $M_T$ could be used to help validate/modify the equation of Furr et
al. [2] in new populations under investigation in this project.

I mentioned above that, as liver stores of vitamin A get large, the vitamin may not
"mix" with the absorbed labeled vitamin A in a reasonable period of time. For the rat,
this problem is illustrated in Fig. 10 which shows data from all of the vitamin A turnover
studies conducted in my laboratory to date. As liver vitamin A begins to increase from a depleted state, the total traced mass is greater than liver vitamin A, indicating that extrahepatic stores of vitamin A exist. When liver vitamin A exceeds ~1000 nmol (~80 nmol/g), the total traced mass is less than the chemically-determined amount of vitamin A. Although some of the studies in vitamin A-adequate rats were carried out for 115 d, the tracer had still not mixed with these stores. These observations need to be kept in mind as we apply isotope dilution methods to human populations with widely differing stores of vitamin A in liver and whole body.

Lastly, I feel we need to reexamine the possibility that urine metabolites might be used as markers of vitamin A stores.

To conclude this section, I would like to flag the following features of the methods we have used in rats that would need to be modified to come up with a method that is feasible in humans. Clearly, we will need to administer the dose orally rather than intravenously, and we will need to use stable isotope-labeled vitamin A instead of radioactive retinol. Furthermore, we will need to have high enough tracer enrichment in the dose to follow plasma tracer disappearance for ~120 d. I base this estimate on the kinetic analysis I performed on the data of DeWitt Goodman (Fig. 4) on vitamin A turnover in 3 men [9]. Also, we must determine the minimum number of plasma samples and the optimal sampling times for obtaining an accurate portrait of the plasma isotope response curve. For this, one can apply sensitivity analysis [16]. In addition, it will be necessary to measure plasma retinol concentration at several selected sampling times, and to analyze a sufficient number of plasma samples collected during the period of dose absorption (e.g., 0.5 - 8 h post dosing) for labeled retinol versus retinyl esters. I also suggest that we use model-based compartmental analysis to analyze the data and calculate total traced mass of vitamin A rather than empirical analysis (multiexponential curve fitting). Finally, I feel that the experiments we are planning will be significantly better if we strive to keep involved, or recruit, scientists with expertise in isotope dilution methods (e.g., Harold Furr), as well as researchers who are experts in tracer kinetics, mathematical modeling and advanced regression methods.

4. ISOTOPE DILUTION ISSUES RELATED TO PREGNANCY AND LACTATION

There is no doubt that the physiological state of the field study subjects will complicate the application of isotope dilution methods to assess vitamin A status. One important fact that we will need to be mindful of is that pregnant/lactating women, and nursing children, are in a non-steady/variable state with respect to vitamin A. The biokinetic behavior of an orally administered stable isotope will be affected by the tracee’s non-steady state.

Further, I predict that at least two features of normal vitamin A metabolism will be altered in pregnancy/lactation and thus need forethought. First, we need to have more information on the role of chylomicrons versus retinol-binding protein as alternative vehicles for delivery of vitamin A to fetus and milk. Second, it is likely that whole-body utilization of vitamin A will be influenced because of transfer of the vitamin to fetus and milk. These processes and their impact on the isotope dilution method need to be investigated. Finally, the choice of dosing regimen (a single large oral dose versus multiple smaller doses) needs to be considered in view of the possibility [22] that a large acute oral load has the potential to be teratogenic to the developing fetus. It is possible that chylomicron delivery of vitamin A, which will be proportional to dietary vitamin A intake, may be responsible for a less well-regulated delivery of vitamin A to the developing fetus.
5. CONCLUDING THOUGHTS

In summary, while some form of the isotope dilution method or a related kinetic evaluation is currently our best bet for a practical and accurate technique for assessing vitamin A status in the field, there are many important and challenging issues that need to be addressed, preferably before field studies are begun. I believe that it will be possible to meet these challenges and that, in so doing, we will have developed a technique for determining vitamin A status that has wide and useful application in humans.

ACKNOWLEDGMENTS

Research conducted in my laboratory and cited in this paper was supported by USDA Competitive Research Grants 81-CRCR-1-0702 and 88-37200-3537; work at the University of Oslo was supported by a Fulbright Research Scholar Award, National Science Foundation Grants INT-8419955 and INT-8619806 and a NATO Grant for International Collaboration. I acknowledge the research assistance of J.B. Green and her help in preparing this manuscript.

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Fig. 1. Schematic of vitamin A metabolism as drawn by Blomhoff [8]. RE, retinyl esters; ROH, retinol; CM, chylomicron; CMR, chylomicron remnant; RBP, retinol-binding protein; TTR, transthyretin; RA, retinoic acid.
Fig. 2. Temporal disappearance of $[^3]$Hretinol from plasma of rats at four levels of vitamin A nutriture. Rats had either high liver vitamin A stores (~9700 nmol) and consumed a low vitamin A-containing diet such that they were in a negative vitamin A balance (inverted triangles); or a liver vitamin A level of ~500 nmol at the time of administration of $[^3]$Hretinol and an intake that resulted in slight positive vitamin A balance so that liver levels increased to ~670 nmol by the end of the 41-day turnover study (boxes); or a starting liver level of ~370 nmol that decreased, as a result of a low vitamin A intake, to ~44 nmol by the end of the study (diamonds); or an initially depleted level of liver vitamin A (~8 nmol) and low plasma retinol concentration (~2 versus ~1 μmol/L in the other groups) both of which increased by the end of the turnover study (to ~18 nmol in the liver) even though rats were consuming the same intake as those depicted by [diamonds]. Lines are fits to the data using a three- or four compartment model. Inset shows data for the first day. Data are from Refs. 12 and 13.
Fig. 3. Model-predicted recycling of $[^3]H$retinol to plasma from extravascular pools. The plasma disappearance data shown in Fig. 2 for rats with "depleted" or "high" liver vitamin A stores were fit to a 3-compartment model (lower panel). Recycling from the fast- and slow turning-over extravascular compartments was simulated (upper panel).
Fig. 4. Plasma retinol kinetics in humans. Shown are observed data (symbols) and model-predicted values (lines) for fraction of injected [\textsuperscript{14}C]retinol remaining in plasma versus time after dose administration. The model is shown in Fig. 5. This figure is reproduced from Ref. 9, Fig. 1.

Fig. 5. Compartmental model for retinol kinetics in humans. Compartments are represented by circles; estimated compartment masses (mg) are shown inside each circle. Asterisk and stippled triangle show site of input of [\textsuperscript{14}C]retinol and site of sampling, respectively; U(2) is the estimated input rate for absorbed dietary vitamin A. Fractional transfer coefficients ± the population estimate of the standard deviation (d\textsuperscript{-1}) and transfer rates (mg/d) are shown above and below each arrow, respectively. This figure is reproduced from Ref. 9, Fig. 2.
Fig. 6. Fraction of an oral dose of $[^3]$Hretinol recovered in plasma versus time after dose administration for a randomly-selected rat from groups with deficient, marginal, adequate or high liver vitamin A levels. This figure is reproduced from Ref. 15, Fig. 1.

Fig. 7. Prediction of liver vitamin A from plasma retinol kinetics. Shown are observed values (squares) and predicted data (lines) for liver total retinol versus the fraction of an oral dose of $[^3]$Hretinol in plasma at 3 days. This figure is reproduced from Ref. 15, Fig. 2.
Fig. 8. Relationship between plasma retinol and disposal rate in rats with liver vitamin A levels ranging from 1 to 11,000 nmol. Data are from Ref. 13.
Fig. 9. Relationship between vitamin A disposal rate calculated from compartmental analysis of plasma \([^3H]\)retinol disappearance data and that predicted from a multiple regression equation (see text) relating plasma retinol, vitamin A intake and liver vitamin A mass. Data are from Ref. 13.

Fig. 10. Relationship between liver vitamin A and model-predicted total traced mass minus liver vitamin A. Data are from Ref. 13.
1. ABSORPTION OF CAROTENOIDS

Several processes are necessary for optimal absorption of carotenoids:

a. sufficient digestion of the food matrix to release carotenoids;
b. formation of lipid micelles in the small intestine;
c. uptake of carotenoids by intestinal mucosa;
d. transport of carotenoids or metabolic products to the lymphatic and/or portal circulation

1.1. Digestion

Absorption of β-carotene from raw, uncooked vegetables can be very low (in the order of 1-2%). Particle size of uncooked foods is particularly important; β-carotene absorption from pureed or finely chopped vegetables is considerably higher than from whole or sliced raw vegetables. Cooking procedures (boiling/steaming) improves the chemical extractability of carotenoids from foods and also appears to improve absorption. This is partly a result of the disruption of the plant cell walls and increased digestibility. Excessive and prolonged heating, however, particularly in the presence of oxygen can result in oxidation of β-carotene with a subsequent loss of vitamin A activity.

1.2. Dietary fat and the formation of lipid micelles

Dietary fat stimulates bile flow from the gall bladder which facilitates the emulsification of fat and fat soluble dietary components into lipid micelles within the small intestine. Without micelle formation carotenoids are very poorly absorbed. Several studies have shown that the absence of dietary fat or very low fat diets substantially reduces β-carotene absorption in human volunteers. For example, Roels et al. [1] showed that in boys with vitamin A deficiency in Rwandan village, supplementation of their carotene-sufficient but low fat diet (about 7% energy) with 18 mg/day of olive oil improved carotene absorption from 5% to 25%.

The disruption of micelle formation with bile acid sequestering drugs also reduces absorption and it has been suggested that some forms of dietary fibre may also inhibit carotenoid utilization, perhaps by sequestering bile salts and reducing lipid micelle formation. Gastrointestinal malabsorption, intestinal parasites and Steatorrhea are also likely to affect carotenoid absorption.

1.3. Uptake of carotenoids by mucosal cells

Carotenoid absorption is thought to be a passive process. The assumption is that carotenoids within lipid micelles come into contact with the intestinal epithelial cell membranes and that transport from micelles to the plasma membrane and cytosol of the cell occur together with the transport of fatty acids. β-carotene appears simultaneously in lymph with newly absorbed fat from a meal and thus it is assumed that they move together across the plasma membrane and within the mucosal cell.
There is still a possibility that there are specific transport mechanisms for carotenoid transport although none have been found and, because many carotenoids in food differ only slightly in structure, it is also possible that there may be competition between them for absorption and subsequent intracellular transport. In ferrets, canthaxanthin appears to reduce β-carotene absorption whilst in one human study (performed by one of my colleagues at the TNO Institute in the Netherlands using the chylomicron response technique) lutein (found in green/yellow vegetables) appeared to reduce both the uptake and cleavage of β-carotene. The levels of lutein and β-carotene given (simultaneously) to the volunteers were approximately 15 mg of each carotenoid. Please see next section on the use of the 'chylomicron response curves' for further details of studies of carotenoid absorption from single test doses or meals.

1.4. Transport

Humans, unlike many other mammals, can cleave pro-vitamin A carotenoids to vitamin A within the intestinal mucosa cells or they can absorb a whole variety of carotenoids intact. Most species do not absorb carotenoids intact.

The control mechanisms regarding mucosal cell uptake, cleavage and transport are poorly understood. It appears from numerous animal studies that as dietary β-carotene intake increases, the efficiency of conversion to vitamin A decreases. This suggests that regulatory mechanisms are in place to limit mucosal uptake, cleavage and/or transport when dietary provitamin A carotenoids are high and when vitamin A status is adequate.

The recommended dietary allowances for preformed vitamin A and the pro-vitamin A carotenoids have been established assuming that carotenoids are less well absorbed than vitamin A and are inefficiently converted to vitamin A. Comparison of the ability of synthetic β-carotene and all-trans retinyl acetate to alleviate vitamin A deficiency in humans demonstrate that 2 µg of β-carotene was equivalent to 1 µg of retinol. Thus with highly absorbed β-carotene we could assume a 2 to 1 weight to weight conversion efficiency. However, the conversion factor of 6 to 1 for β-carotene and 12 to 1 for other provitamin A carotenoids is used for dietary carotenoids which are more poorly absorbed. It is obvious, however, considering the comments in Section 1.1 that this factor will be highly variable depending on digestibility, particle size, cooking procedure and physiological state of the consumer - and possibly other factors.

1.5. Metabolism

The metabolism of absorbed carotenoid has not been studied in detail and is not well understood. Humans transport carotenoids in blood plasma via lipoproteins, the majority of β-carotene being associated with the low density lipoprotein (ca. 75%). Generally the appearance of β-carotene in the LDL follows the same time course as newly absorbed triglycerides.

Oral doses of β-carotene (up to 120 mg) have been administered to human volunteers and plasma response determined. In some individuals a rise in plasma β-carotene is observed but other subjects showed no increase in plasma β-carotene concentration - these subjects have been classified as non-responders. I prefer to think of individuals responding differently rather than classifying them as responders and non-responders. We know so little about the control and rates of carotenoid absorption, distribution and utilization that the best we can say at present is that subjects under a particular set of experimental conditions, and on that occasion, did or did not show an increase in plasma β-carotene concentration, over a specified time-course, after a specified dose. It is also very important that the physiological state of the subject be taken into consideration. We at IFR, and others, have information which would point to an inverse relationship between plasma
carotenoid concentration and body mass index. The adiposity of an individual may therefore influence the rate of clearance of carotenoids from the plasma. There is also evidence to suggest that the 'residence time' of carotenoids in the mucosal cell, mucosal cell turnover rate and re-uptake of carotenoids from mucosal cells sloughed off into the intestinal lumen may all influence the rate of appearance of carotenoids in the plasma. In some cases a small surge of \( \beta \)-carotene can be seen in chylomicrons following a carotene-free meal given subsequent to a test dose.

2. CHYLOMICRON RESPONSE

In humans, chylomicrons (MC) and very low density lipoprotein (VLDL), composed mainly of triglycerides are packaged in the intestine and transported through the lymphatic system to the thoracic duct. This then empties into the subclavian vein and enters the main circulation. Lipoprotein lipase acts upon the CM by hydrolysing core triglycerides, resulting in the formation of a CM remnant. The CM remnants are then taken up by the liver where the carotenoids are utilized to form vitamin A and /or stored or repackage and released with VLDL particles. In humans, CM and VLDL \( \beta \)-carotene levels peak around 4 to 8 hours following a \( \beta \)-carotene dose. As these are cleared by the liver, LDL \( \beta \)-carotene levels start to rise with a peak level at 24-48 hours post dosing, while HDL levels peak around 16 to 48 hepatic hours post dosing. It is unclear whether there is transfer of carotenoids from CM to extra hepatic tissues prior to uptake of CM remnants by the liver, nor is it known what factors are important in determining the uptake of carotenoids by tissues and their recycling back into the plasma.

In most human studies of carotenoid absorption plasma or serum response curves are used as a measure of carotenoid uptakes (see previous section). However, the problem with such profiles is that no discrimination can be made between exogenous and endogenous carotenoids and retinoids. In recent studies at TNO in the Netherlands (as part of an EC funded project coordinated by IFR) the measurement of carotenoid and retinyl ester (RE) response in isolated chylomicron fractions, obtained at various times after an oral dose, has been studied, as a potentially more appropriate measure of carotenoid absorption and cleavage. The carotenoid/retinyl ester response must be corrected for triglyceride response. After such correction, the within person variability in both the carotenoid and RE response (as area under the curve) was about 20% (as CV).

To determine the CM response curve, volunteers are requested to consume a diet with restricted vitamin A and carotenoids for one week before each experiment (through dietary advice and written instructions). Two days before the actual experiment volunteers are given a complete, standardised vitamin A-free/low carotenoid diet provided by the research centre. The test dose or food item is then given after an overnight fast. A fasting (time 0) blood sample is collected and 8 postprandial blood samples obtained over 10 hours after the dose. Blood is obtained via an indwelling catheter inserted into the anticubital vein. Plasma is separated by centrifugation and CM fractions by further density gradient centrifugation. Approximately 14 ml of blood is taken at each time point (the volume of blood can be reduced depending on what analyses are to be performed). Approximately 3 ml of plasma is required for separation of chylomicrons; 0.5 ml for triglyceride analysis and 1 to 3 ml (depending on the method of analysis) of plasma for carotenoid and retinyl ester analysis.

So far in the EC studies, the following test doses have been administered: palm oil carotenoids (15 mg total \( \alpha \) and \( \beta \)-carotene); combined palm-oil carotenes and lutein (15 mg of each); 15 mg lycopene; combined palm-oil carotenes and lycopene (15 mg of each). These carotenoids were given as test meals consisting of the carotenoid dispersed in skimmed yoghurt with added sugar and with 50 g arachis oil to trigger fat digestion and
absorption. Each volunteer received each test dose in a randomized cross-over design with a three week wash-out between each period.

In addition, to assess the feasibility of postprandial CM response curves as a measure of absorption and cleavage of carotenoids from vegetables an additional ‘dose’ of carotenoids was given as a portion of vegetable or fruit with peanut oil and sugar. The following vegetables have been used: carrots boiled for 20 min. in an amount to provide 15 mg β-carotene; boiled spinach to provide 15 mg lutein and fresh tomato paste containing 15 mg lycopene. There were 4 volunteers for each vegetable meal.

Differences in the areas under the chylomicron response curves (AUC) are statistically assessed using analysis of variance.

After each of these treatments the triglyceride CM response curves were not significantly different confirming that the test protocol is reproducible with acceptable within-individual variability in fat response. However, for the carotenoids a wide range of plasma and chylomicron response was observed both within and between individuals. A CM response was observed for all the test doses of isolated carotenoids. Preliminary analysis indicates that when lutein was administered with the palm oil the area under the CM response curve for both β-carotene and retinyl esters was reduced as compared to the single dose of palm oil. Combined dosage of palm-oil with lycopene had no apparent effect.

When the vegetable preparations were given, only the tomato paste (lycopene) showed a measurable CM response. In the case of carrots and spinach no significant response could be demonstrated for α- and β-carotene and lutein, respectively. This is puzzling. We have shown from dietary intake studies that the plasma concentration of specific carotenoids is significantly correlated to intake of fruit and vegetable sources of that carotenoid, although it must be said that the weakest correlation (although still significant) was for β-carotene. We had assumed that this was because of its losses via cleavage. At present, with so little knowledge of the factors which affect total amounts of carotenoids absorbed from our food, the rates of that absorption and differences in chylomicron clearance from a ‘slug’ of readily and quickly absorbed carotenoid versus a food source of an equal amount which may produce a much longer and shallower response, it cannot be assumed that the lack of a CM response equates to a lack of absorption. Further studies are underway to examine the phenomenon more closely for example, to consider the physico-chemical factors (e.g. effect of food preparation and processing) on CM appearance of the carotenoids. An indication that this might be important comes from the measurable lycopene CM response from tomato paste - a pureed, heat treated, thin cell walled, easily extractable product. In order to develop some sustainable food-based interventions in vitamin A deficient populations such information is important eg is it better to find some soft-cell walled fruit source of provitamin A carotenoids rather than vegetable sources?

3. THE EFFECT OF β-CAROTENE SUPPLEMENTATION ON THE IMMUNE FUNCTION OF BLOOD MONOCYTES FROM HEALTHY MALE NON-SMOKERS

3.1. Background and aims of the study

There is strong epidemiological evidence that diets rich in fruits and vegetables are associated with reduced incidence of cancer. Amongst the many compounds in fruits and vegetables that prevent cancer in laboratory animals, attention has been focused on the carotenoids, a group of highly pigmented, fat-soluble antioxidants. One of these compounds, β-carotene, has been shown to be particularly effective in preventing cancer in animal models and many epidemiological studies have shown a strong inverse association between intake of β-carotene and incidence of cancer. However, these types of studies provide little
information on biological mechanisms. The immune system plays a major role in cancer prevention by its involvement in a variety of anti-tumour effector mechanisms and it has been suggested that one mechanism by which β-carotene can prevent carcinogenesis is by the enhancement of immune cell activity. Cell mediated immune responses are initiated by the stimulation of appropriate T lymphocytes by antigen-presenting cells. A pre-requisite for this antigen-presenting cell function is the expression of major histocompatibility complex (MHC) class II molecules (HLA-DR, HLA-DPP and HLA-DQ), which are present on the majority of human monocytes, macrophages and dendritic cells (see figure). Since the degree of immune responsiveness of an individual has been shown to be proportional to both the percentage of MHC class II-positive monocytes and the density of these molecules on the cell surface, it is possible that one mechanism by which β-carotene may enhance cell mediated immune responses is by enhancing the cell surface expression of these molecules.

In addition, cell to cell adhesion appears to be critical for the initiation of a primary immune response, and it has recently been shown that the intercellular adhesion molecule-1 (ICAM-1)-leucocyte function associated antigen-1 (LFA-1) ligand-receptor pair is also capable of co-stimulating an immune response, enhancing T cell proliferation and cytokine production. Therefore, we have examined the effect of β-carotene supplementation, at a dietarily achievable level, on the cell surface expression on blood monocytes of MHC class II molecules and of several adhesion molecules known to be involved in the process of antigen presentation.

Since the ability to mount an immune response is also dependent on communication between different immune cell types, some insight into the influence of different nutrients on the immune system can be gained by studying their effects on the production of cytokines, polypeptide messenger molecules released by a variety of cell types which play a major role in initiating and sustaining immune responses. Tumour necrosis factor-alpha (TNF-α) is an immunostimulatory cytokine, secreted primarily by blood monocytes and tissue macrophages, which has a selective toxic effect on a variety of malignant cells and can also initiate the cascade of cytokines and other factors associated with inflammatory responses. Therefore, it is thought that this molecule plays a critical role in normal host resistance to infections and to the growth of malignant tumours. It has been demonstrated that regression of established hamster buccal pouch carcinoma following the local injection of β-carotene is associated with an induction of TEF-α in macrophages at the tumour site. It has also been shown in vitro that β-carotene can increase the stimulated secretion of TEF-α by human peripheral blood mononuclear cells. Therefore, we have also investigated whether supplementation with β-carotene can enhance the ability of human blood monocytes to secrete TEF-α when stimulated ex vivo.

3.2. Methods and results

The study design, participant details and results are presented in the following tables, and were discussed during the RCM. Figure 1 shows the outline of the study design. Tables I to VIII depict clinical and biochemical findings.

3.3. Conclusions

We conclude that a 4-week supplementation with β-carotene, at dietarily achievable levels, was associated with an elevated expression of cell surface molecules on blood monocytes which are involved in initiating immune responses. This might be one mechanism where β-carotene can cause enhancement of immune responsiveness and, consequently, of tumour surveillance.

We have recently commended a comparable human supplementation study with lycopene.
The following is the assay for natural killer cell function (as promised at the RCM). It requires rather a lot of blood and is probably not a practical option for most of the studies. Nevertheless all details of the assay are attached and if there is any further interest please do not hesitate to contact either Sue Southon or David Hughes at the Institute of Food Research.

4. NATURAL KILLER (NK) CELL CYTOTOXICITY ASSAY

4.1. Target cells

K562, a human chronic myelogenous leukaemic cell line, obtainable from the European Collection of Animal Cell Cultures (ECACC No. 89121407).

Cells cultured in RPMII-1640 supplemented with 10% heat inactivated fetal calf serum (FCS), in 50 ml tissue culture flasks kept in an upright position. The cells grow in free suspension. Incubate at 37°C in 5% CO₂.

Maintain by subculture twice a week, splitting approximately 1:3 (to final volume of 20 ml), and subculture on the day prior to the assay.

4.2. Effector cells

Approximately 3 X 10⁶ cells are required for this assay, although the number can be reduced if the assay is performed in triplicate rather than quadruplicate. Therefore, approximately 5-7 ml of blood should be taken per subject.

Try to include a healthy adult subject within each assay “control” to confirm that the assay is working correctly.

Separate peripheral blood mononuclear cells (PB MN) using Ficoll-Hypaque technique. Wash cells twice in Minimal Essential Medium (MEM) and adjust to 2 x 10⁶ cells/ml in RPMI with 10% FCS.

Either use these cells or, for more accurate determinations, incubate PB MN overnight in 50 ml culture flasks, to remove adherent monocytes from the assay. Aspirate off peripheral blood lymphocytes (PBL), wash twice in RPMI, and adjust to 2 x 10⁶ cells/ml in RPMI supplemented with 10% FCS.

4.3. Assay

a. Spin down 2 flasks of K562 cells. Wash twice in RPMI. Adjust to 2 x 10⁶ cells/ml in RPMI.

b. Incubate 1 ml of K562 with 3.7 MBq of Chromium (Cr) for 1 hour at 37°C in a sealed plastic tube placed in a lead pot. (You need to calculate the volume of Cr to add from the activity chart provided with the Cr, due to the short half-life of Cr).

c. Make serial dilutions of effector cells: Take 0.3 ml of cells at 2 x 10⁶/ml (Conc a) and add to 1.2 ml RPMI - 5 x 10⁵/ml (Conc B).

d. Take 0.3 ml of cells at 5 x 10⁵/ml and add to 0.3 ml RPMI = 2.5 x10⁵/ml (Conc C).
e. Take the incubated K562 cells and wash 3 times in RPMI, the last wash immediately prior to use. Suspend the final cell pellet in 1 ml of RPMI and take 0.15 ml and add to 5 ml RPMI = 5 x 10^4 cells/ml (make up a larger volume of cells if doing more than 3 subjects at a time).

f. Also require Triton x100 detergent (Sigma) to lyse cells to indicate maximal Cr release.

g. Set up round bottomed microtitre plate as follows:

<table>
<thead>
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<th>Row</th>
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<td>RPMI only</td>
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<td>x100 100μl</td>
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<tr>
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<td>200 μl</td>
<td>K562 100μl</td>
<td>K562 100μl</td>
</tr>
<tr>
<td></td>
<td>(Background)</td>
<td>(Spontaneous release)</td>
<td>(Maximal release)</td>
</tr>
<tr>
<td>2</td>
<td>Control PBL 100μl</td>
<td>Control PBL 100μl</td>
<td>Control PBL 100μl</td>
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<tr>
<td></td>
<td>Conc C</td>
<td>Conc B</td>
<td>Conc A</td>
</tr>
<tr>
<td></td>
<td>K562 100μl</td>
<td>K562 100μl</td>
<td>K562 100μl</td>
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<td>Subject 1 PBL 100μl</td>
<td>Subject 1 PBL 100μl</td>
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<tr>
<td></td>
<td>Conc C</td>
<td>Conc B</td>
<td>Conc A</td>
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<td></td>
<td>K562 100μl</td>
<td>K562 100μl</td>
<td>K562 100μl</td>
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<td>Subject 2 PBL 100μl</td>
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<td>Conc B</td>
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<tr>
<td></td>
<td>K562 100μl</td>
<td>K562 100μl</td>
<td>K562 100μl</td>
</tr>
</tbody>
</table>

h. Incubate late at 37°C for 4 hours in a humidified atmosphere.

i. Harvest the supernatants by either centrifuging the plate and aspirating the samples, or purchase an automatic harvester from Skatron Instruments Ltd. (Approx. £600 including disposables; address available if required). This instrument is very useful both in terms of convenience and containment of radioactive waste. Measure the radioactivity of the sample in a gamma counter.

j. Calculate % cytotoxicity at each ratio of effector:target cells:-

NB. Try to ensure that the cpm spontaneous release is <15% of cpm maximal release.

Queries to: Dr. David a. Hughes
Institute of Food Research
Norwich Research Park
Colney, Norwich, NR4 7UA
UK
Tel.: +44 01603 255345
Fax: +44 01603 507723
<table>
<thead>
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<th>Group (2 = 11)</th>
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<td>Mean  SEM  Range</td>
<td>Mean  SEM  Range</td>
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<tr>
<td>Age (yrs)</td>
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<tr>
<td>Height (m)</td>
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<td>Weight (kg)</td>
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<td>79.8  2.2  61.2-100.0</td>
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<td>Body Mass Index (BMI)</td>
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<td>24.8  1.2  19.2-31.4</td>
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<td>Haemoglobin (g/l)</td>
<td>152.1 2.2 138.0-166.0</td>
<td>154.9 2.0 143.0-163.0</td>
<td>153.3 1.5 138.0-166.0</td>
</tr>
<tr>
<td>Fasting blood glucose (mmol/l)</td>
<td>3.68 0.10 2.90-4.65</td>
<td>3.55 0.07 3.20-4.00</td>
<td>3.62 0.07 2.90-4.65</td>
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Means within each row were subjected to one-way "analysis of variance". There were no significant (P<0.05) "variance ratios (F)" and therefore no differences between means.
<table>
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<tr>
<th>Parameter</th>
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<td>Range</td>
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<td>Uric acid (µmol/l)</td>
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<td>13.0</td>
<td>276.1-455.8</td>
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<tr>
<td>Retinol (ng/ml)</td>
<td>644.1</td>
<td>29.4</td>
<td>422.4-824.0</td>
</tr>
<tr>
<td>A-tocopherol (ng/ml)</td>
<td>11637</td>
<td>859</td>
<td>4665-16341</td>
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</table>

Means within each row were subjected to one-way 'analysis of variance'. There were no significant (P<0.05) 'variance ratios (F)' and therefore no differences between means.
TABLE III. BASELINE PLASMA CONCENTRATIONS OF CAROTENOIDS, LUTEIN, ZEAXANTHIN, B-CRYPTOXANTHIN, LYCOPENE, A-CAROTENE, TRANS-B-CAROTENE AND TOTAL-B-CAROTENE (TRANS-B-CAROTENE + CIS-B-CAROTENE).

<table>
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<th>Parameter</th>
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</thead>
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<td>Trans-b-carotene (ng/ml)</td>
<td>218.3</td>
<td>29.0</td>
<td>91.1-445.8</td>
</tr>
<tr>
<td>Total-b-carotene (ng/ml)</td>
<td>227.7</td>
<td>31.0</td>
<td>91.1-477.0</td>
</tr>
</tbody>
</table>

Means within each row were subjected to one-way 'analysis of variance'. There were no significant (P<0.05) 'variance ratios (F)' and therefore no differences between means.
**TABLE IV. MONOCYTE CELL-SURFACE MAKERS; MHC CLASS II MOLECULES (HLA-DR, HLA-DP & HLA-DQ) AT BASELINE (B), AFTER 4 WEEKS TREATMENT WITH PLACEBO (P) OR AFTER 4 WEEKS TREATMENT WITH A BETA CAROTENE (15 MG/D) SUPPLEMENT (S). PERCENTAGE +VE MONOCYTES* (%POS); MEDIAN FLUORESCENCE INTENSITY* (MFI).**

<table>
<thead>
<tr>
<th>Group (n:Treat)</th>
<th>Baseline (B)</th>
<th>4 weeks treatment</th>
<th>4 weeks treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parameter</td>
<td>Group 1 = Initial</td>
<td>Group 1 = Placebo</td>
</tr>
<tr>
<td>1. (n = 10; B-P-S)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HLA-DR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>(% pos)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>78.2*</td>
<td>6.2</td>
<td>54.3-88.3</td>
</tr>
<tr>
<td>2</td>
<td>68.9*</td>
<td>7.6</td>
<td>40.3-96.0</td>
</tr>
<tr>
<td></td>
<td>(mfi)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>61.8</td>
<td>4.8</td>
<td>33.0-96.6</td>
</tr>
<tr>
<td>2</td>
<td>49.1</td>
<td>4.7</td>
<td>25.2-68.3</td>
</tr>
<tr>
<td></td>
<td>HLA-DP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>(% pos)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>36.4</td>
<td>6.3</td>
<td>22.0-67.4</td>
</tr>
<tr>
<td>2</td>
<td>26.5</td>
<td>4.7</td>
<td>12.0-49.9</td>
</tr>
<tr>
<td></td>
<td>(mfi)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>26.7</td>
<td>3.0</td>
<td>10.7-36.3</td>
</tr>
<tr>
<td>2</td>
<td>18.3</td>
<td>2.3</td>
<td>8.6-29.4</td>
</tr>
<tr>
<td></td>
<td>HLA-DQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>(% pos)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20.2</td>
<td>4.4</td>
<td>2.6-41.1</td>
</tr>
<tr>
<td>2</td>
<td>15.9</td>
<td>3.8</td>
<td>3.4-37.5</td>
</tr>
<tr>
<td></td>
<td>(mfi)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>15.1</td>
<td>2.6</td>
<td>5.6-29.7</td>
</tr>
<tr>
<td>2</td>
<td>11.7</td>
<td>1.9</td>
<td>3.6-23.9</td>
</tr>
</tbody>
</table>

Means within each row were subjected to one-way analysis of variance for 'repeated measures'. Where the variance ratio (F) was significant, means were then compared by 1-tail paired t-test.

A,B,C = group 1; X,Y,Z = group 2. Means, within a row, having different superscripts are significantly different (P<0.05).
TABLE VII. PLASMA CHOLESTEROL, ASCORBIC ACID, URIC ACID, RETINOL AND A-TOCOPHEROL; AT BASELINE (B), AFTER 4 WEEKS TREATMENT WITH PLACEBO (P) OR AFTER 4 WEEKS TREATMENT WITH BETA-CAROTENE (15 MG/D) SUPPLEMENT (S).

<table>
<thead>
<tr>
<th>Group (n:Treat)</th>
<th>Baseline (B)</th>
<th>4 weeks treatment Group 1 = Placebo</th>
<th>4 weeks treatment Group 1 = Supplement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1 = Initial</td>
<td>Group 1 = Placebo</td>
<td>Group 1 = Supplement</td>
</tr>
<tr>
<td></td>
<td>Group 2 = Initial</td>
<td>Group 2 = Supplement</td>
<td>Group 2 = Placebo</td>
</tr>
<tr>
<td>Parameter</td>
<td>Group</td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1</td>
<td>5.71</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.35</td>
<td>0.31</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>1</td>
<td>55.10</td>
<td>5.32</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>62.64</td>
<td>7.50</td>
</tr>
<tr>
<td>Uric acid</td>
<td>1</td>
<td>346.9*</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>371.7*</td>
<td>18.0</td>
</tr>
<tr>
<td>Retinol</td>
<td>1</td>
<td>644.1</td>
<td>29.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>712.5</td>
<td>57.6</td>
</tr>
<tr>
<td>A-Tocopherol</td>
<td>1</td>
<td>11637</td>
<td>859</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>12216</td>
<td>1013</td>
</tr>
</tbody>
</table>

Means within each row were subjected to one-way analysis of variance for 'repeated measures'. Where the variance ratio (F) was significant, means were then compared by 1-tail paired t-test.

A,B,C = group 1; X,Y,Z = group 2. Means, within a row, having different superscripts are significantly different (P<0.05).
TABLE VI. MONOCYTE UNSTIMULATED (U-) AND LIPOPOLYSACCHARIDE-STIMULATED (S-) SECRETION OF TUMOR NECROSIS FACTOR (TNF-α), AND PROPORTION OF DNA IN UNTREATED (U-) AND HYDROGEN PEROXIDE TREATED (TR-) WBC NUCLEI ‘COMET’ TAILS; AT BASELINE (B), AFTER 4 WEEKS TREATMENT WITH PLACEBO (P) OR AFTER 4 WEEKS TREATMENT WITH BETA-CAROTENE (15 MG/D) SUPPLEMENT (S).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group (n:Treat)</th>
<th>Baseline (B)</th>
<th>4 weeks treatment</th>
<th>4 weeks treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Group 1 = Initial</td>
<td>Group 1 = Placebo</td>
<td>Group 1 = Supplement</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Group 2 = Initial</td>
<td>Group 2 = Placebo</td>
<td>Group 2 = Supplement</td>
</tr>
<tr>
<td>U-TNF-α (ng/2x10⁶ cells)</td>
<td>1</td>
<td>0.604* 0.061</td>
<td>0.410-1.283</td>
<td>0.658* 0.089</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.576* 0.046</td>
<td>0.377-0.910</td>
<td>1.117* 0.499</td>
</tr>
<tr>
<td>S-TNF-α (ng/2x10⁶ cells)</td>
<td>1</td>
<td>3.274* 0.513</td>
<td>0.623-6.957</td>
<td>5.376* 1.278</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.724* 0.485</td>
<td>0.817-5.837</td>
<td>4.955* 1.193</td>
</tr>
<tr>
<td>U-Comet (tail DNA/total)</td>
<td>1</td>
<td>0.294* 0.029</td>
<td>0.181-0.557</td>
<td>0.368* 0.013</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.237* 0.018</td>
<td>0.143-0.340</td>
<td>0.419* 0.028</td>
</tr>
<tr>
<td>TR-Comet (tail DNA/total)</td>
<td>1</td>
<td>0.613* 0.036</td>
<td>0.390-0.821</td>
<td>0.615* 0.063</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.519* 0.052</td>
<td>0.285-0.713</td>
<td>0.470* 0.028</td>
</tr>
<tr>
<td>Change in Comet (treat - untreated)</td>
<td>1</td>
<td>0.319* 0.034</td>
<td>0.114-0.501</td>
<td>0.215* 0.063</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.282* 0.044</td>
<td>0.054-0.423</td>
<td>0.051* 0.023</td>
</tr>
</tbody>
</table>

Means within each row were subjected to one-way analysis of variance for ‘repeated measures’. Where the variance ratio (F) was significant, means were then compared by 1-tail paired t-test.
A,B,C = group 1; X,Y,Z = group 2. Means, within a row, having different superscripts are significantly different (P<0.05).
### TABLE VII. PLASMA CHOLESTEROL, ASCORBIC ACID, URIC ACID, RETINOL AND A-TOCOPHEROL; AT BASELINE (B), AFTER 4 WEEKS TREATMENT WITH PLACEBO (P) OR AFTER 4 WEEKS TREATMENT WITH BETA-CAROTENE (15 MG/D) SUPPLEMENT (S).

<table>
<thead>
<tr>
<th>Group (n:Treat)</th>
<th>Baseline (B)</th>
<th>4 weeks treatment</th>
<th>4 weeks treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1. (n=14; B-P-S)</td>
<td>Group 1 = Initial</td>
<td>Group 1 = Placebo</td>
</tr>
<tr>
<td></td>
<td>2. (n=11; B-S-P)</td>
<td>Group 2 = Initial</td>
<td>Group 2 = Supplement</td>
</tr>
<tr>
<td>Parameter</td>
<td>Group</td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1</td>
<td>5.71</td>
<td>0.29</td>
</tr>
<tr>
<td>(mmol/l)</td>
<td>2</td>
<td>5.36</td>
<td>0.31</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>1</td>
<td>55.10</td>
<td>5.32</td>
</tr>
<tr>
<td>(μmol/l)</td>
<td>2</td>
<td>62.64</td>
<td>7.50</td>
</tr>
<tr>
<td>Uric acid</td>
<td>1</td>
<td>346.9</td>
<td>13.0</td>
</tr>
<tr>
<td>(μmol/l)</td>
<td>2</td>
<td>371.7</td>
<td>18.0</td>
</tr>
<tr>
<td>Retinol</td>
<td>1</td>
<td>646.1</td>
<td>29.4</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td>2</td>
<td>712.5</td>
<td>57.5</td>
</tr>
<tr>
<td>A-Tocopherol</td>
<td>1</td>
<td>11637</td>
<td>859</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td>2</td>
<td>12216</td>
<td>1013</td>
</tr>
</tbody>
</table>

Means within each row were subjected to one-way analysis of variance for 'repeated measures'. Where the variance ratio (F) was significant, means were then compared by 1-tail paired t-test.

A, B, C = group 1; X, Y, Z = group 2. Means, within a row, having different superscripts are significantly different (P<0.05).
TABLE VIII. PLASMA CONCENTRATIONS OF CAROTENOID, LUTEIN, ZEAXANTHIN, B-CRYPTOXANTHIN, LYCOPENE, A-CAROTENE, TRANS-B-CAROTENE AND TOTAL-B-CAROTENE (TRANS-B-CAROTENE + CIS-B-CAROTENE); AT BASELINE (B), AFTER 4 WEEKS TREATMENT WITH PLACEBO (P) OR AFTER 4 WEEKS TREATMENT WITH BETA CAROTENE (15 MG/D) SUPPLEMENT (S).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Baseline (B)</th>
<th>4 weeks treatment</th>
<th>4 weeks treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Range</td>
</tr>
<tr>
<td>Lutein (ng/ml)</td>
<td>1</td>
<td>130.6&quot;</td>
<td>13.5</td>
<td>41.3-196.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>148.4</td>
<td>8.6</td>
<td>30.4-122.6</td>
</tr>
<tr>
<td>Zeaxanthin (ng/ml)</td>
<td>1</td>
<td>58.4&quot;</td>
<td>9.3</td>
<td>10.6-149.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>56.7&quot;</td>
<td>8.6</td>
<td>30.4-122.6</td>
</tr>
<tr>
<td>B-cryptoxanthin (ng/ml)</td>
<td>1</td>
<td>85.6</td>
<td>14.8</td>
<td>20.9-163.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>71.6</td>
<td>11.1</td>
<td>18.9-130.3</td>
</tr>
<tr>
<td>Lycopene (ng/ml)</td>
<td>1</td>
<td>166.3</td>
<td>26.7</td>
<td>27.5-359.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>154.5</td>
<td>17.9</td>
<td>32.8-246.5</td>
</tr>
<tr>
<td>A-carotene (ng/ml)</td>
<td>1</td>
<td>57.2&quot;</td>
<td>7.7</td>
<td>20.6-122.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>87.6&quot;</td>
<td>28.4</td>
<td>23.4-297.6</td>
</tr>
<tr>
<td>Trans-b-carotene (ng/ml)</td>
<td>1</td>
<td>218.3&quot;</td>
<td>29.0</td>
<td>91.1-445.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>270.0&quot;</td>
<td>73.7</td>
<td>52.7-871.1</td>
</tr>
<tr>
<td>Total-b-carotene (ng/ml)</td>
<td>1</td>
<td>227.7&quot;</td>
<td>31.0</td>
<td>91.1-477.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>293.6&quot;</td>
<td>79.1</td>
<td>52.7-927.9</td>
</tr>
</tbody>
</table>

Means within each row were subjected to one-way analysis of variance for 'repeated measures'. Where the variance ratio (F) was significant, means were then compared by 1-tail paired t-test.

A,B,C = group 1; X,Y,Z = group 2. Means, within a row, having different superscripts are significantly different (P<0.05).
PART III: WORKING PAPERS
ASSESSMENT AND FOOD BASED INTERVENTION OF VITAMIN A STATUS IN
CHINESE CHILDREN

SHIAN YIN¹, GUANGWEN TANG²

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29 Nan Wei Road, Beijing, People’s Republic of China
²USDA Human Nutrition Research Centre for Aging at Tufts University, 711
Washington St., Boston, MA 02111, USA

Abstract

To assess food based intervention of vitamin A status in children, this study
will first identify a geographic area with vitamin A deficiency in a childhood
population. After screening dietary intakes of vitamin A from 300 pre-school
children aged 4-6 years, a stable isotope labeled (non-radioactive) vitamin A
dose will be ingested to those subjects with low dietary vitamin A intakes
(n = 3D48) to determine their liver stores of vitamin A. Their blood sample
will be collected 14 and 25 days after the administration of the dose. Foods
rich in pro-vitamin A carotenoids low in retinoids will then be supplied to 24
subjects in one kindergarten and the regular food low in carotenoids and
retinoids will be supplied to another 24 subjects in another kindergarten for
3 months. Two groups will take equal amounts of retinoids, protein and
energy but carotenoids from their meals. At the end of the study (10 weeks
on supplementation), a second stable isotope labeled vitamin A will be given
and blood samples will be taken.

1. SCIENTIFIC BACKGROUND AND SCOPE OF THE PROJECT

Vitamin A deficiency remains an important public health problem in China, mainly
affecting young children and pregnant women. In 1992, the retinol equivalent intake from
the National Nutritional Survey was 476 µg in average [1]. According to the China National
Wide Nutrition Survey carried out in 1982 [2], the prevalence of vitamin A deficiency was
0.9% among 30 000 subjects. Investigations of serum vitamin A in children showed that
the prevalence of children whose serum vitamin A were below the lower limit of normal
(<7µmol/L) was 37.1%, 39.3% and 17.8%, respectively, in Hubei Province, Tianjing, and
Wuhan countryside. The percentage of urban children in Wuhan, who were vitamin A
deficient, was 10.0% in 1989. As children’s dietary intake of vegetable was lower than the
adults, the insufficiency of vitamin A was found more frequently in the former.

1.1. Dietary pattern and vitamin A nutritional status of the Chinese

Results from the National Nutritional Dietary Surveys carried out in 1982 and 1992,
respectively, demonstrated that the food pattern has improved. Then, 238 134 subjects
in 27 provinces/districts were investigated in 1982 and 27 000 families in 30 provinces and
districts were also investigated in 1992. It showed that cereals and tuber intakes of male
adults decreased in 1992 whereas foods of other variety increased [1,2]. Table I shows

In 1982, an average dietary energy intake of male adult was 10.40 MJ. The average
dietary intakes of protein, fat, vitamin A, and B-carotene were 66.8 g, 49.3 g, 252.9 IU and
3.48 mg (the sum of vitamin A and B-carotene equals to 655 µg RE). In 1992, dietary
pattern of 99 749 subjects in 30 provinces were studied. The retinol equivalent intake was
476 µg RE on average. The Chinese dietary origin of vitamin A was mostly from B-carotene. Before and at the early stage of founding of People's Republic of China, vitamin A deficiency was a serious problem and one of the major causes of blindness in children. With the development of national economy and the extension of medical care and health services to rural areas, the vitamin A nutritional status of population has greatly improved. Hu et al. reported that the prevalence of xerophthalmia was below 0.7%. In recent years there were only conjunctival xerosis cases in winter, but keratomalacia was not found. In 30,000 subjects investigated in National Nutritional Dietary Survey in 1982, the diagnostic criteria for vitamin A deficiency were the positive findings of more than 2 of the following 5 symptoms or signs: i) night blindness; ii) conjunctival xerosis; iii) conjunctival folding; iv) follicular keratosis and v) scaly skin [2]. The prevalence of vitamin A deficiency was 0.9% in the examined subjects. Therefore, vitamin A nutritional status of Chinese people needs to be improved.

1.2. Nutritional status of vitamin A in Chinese children

Chang reported in 1987 that 37.1% of the 97 rural infants and children had serum concentration of vitamin A that was lower than 0.7 µmol/L, 2.1% had serum vitamin A level lower than 0.35 µmol/L (10 µg/dl). Pang reported that the average concentration of serum vitamin A in 30 pre-school children in kindergarten was 0.92 ~ 0.30 µmol/L, and 16.7% of 30 children had a serum vitamin A content that was < 0.7 µmol/L, while 3.3% of 30 children had a serum vitamin A concentration < 0.53 µmol/L [3]. Serum of vitamin A levels of 774 rural children aged from 3 months to 7 years old in Tianjin in 1988 was studied. From which, 39.3% had a serum vitamin A levels < 0.7 µmol/L. Among vitamin A insufficient children, there were positive correlation between serum vitamin A and Hb, serum vitamin A and body height, serum vitamin A and body weight: \( r = 0.222, r = 0.267, r = 0.239, p < 0.01 \).

Most of the rural children with serum vitamin A levels lower than 0.7 µmol/L were aged below 2 years old. The average retinol equivalent intake was 699.6 µg in urban children under 1 year old. Dietary intake of RE in the urban toddlers were 211-284 µg. The dietary RE intake in the rural children was prominently lower than the urban children, especially in the toddlers. Their dietary intakes of RE were only 31.3-37.5% of Chinese RDA. One hundred and eleven pre-school children of 3-6 years old in Wuhan city were examined by Li et al. in 1993 and the number of children with serum vitamin A level > 1.05 µmol/L, 0.7 - 1.05 µmol/L and < 0.7 µmol/L were 91 (81.98%), 19 (17.1) and 1 (0.9%), respectively [4]. The data showed that the nutritional status of children was higher than that of 1989.

Li et al. reported the application of impression cytology method for early detection of vitamin A status in children [4]. A slip of thin cellulose acetate was put on the conjunctiva for 3 sec, and then was fixed, stained, and examined under microscope. Normally, there are globate cells and small epithelial cells. The disappearance of the globate cells and enlargement of the epithelial cells indicates vitamin A deficiency. This investigation showed that 64.3% of children with abnormal impression cytology had low level of serum vitamin A < 1.05 µmol/L (30 µg/dl), while 97.5% of the children with normal conjunctiva impression cytology had serum vitamin A levels > 1.05 µmol/L. This result suggested that impression cytology could identify sub-clinical vitamin A deficiency and might be used in field investigation.

1.3. Impact of large doses of vitamin A supplementation on childhood diarrhea and respiratory diseases

From previous study, we observed that there was a positive impact of large doses of vitamin A supplementation on childhood diarrhea and respiratory diseases [5,6].
hundred and seventy-two of 6 months to 2 years old children were randomly assigned to either participate in a vitamin A supplementation group (n = 98) or serve as control (n = 74) for 1 year. Capsules containing 200 000 IU vitamin A and 40 IU vitamin E were given to the experimental group by the institute's staff 3 months after baseline examination and again 6 months later. During the 12-month study period, there was a significant reduction in the incidence of diarrhea and respiratory diseases in the children of the experimental group as compared to the control group. The risk of diarrhea and respiratory diseases was 2-3 and 3-4 times higher, respectively, in the control group. Eight weeks after first supplementation the serum vitamin A level of the experimental group was higher than that of control group, and the percentage of children whose serum vitamin A was

The Chinese dietary origin of vitamin A is mostly B-carotene. However, the bioavailability of B-carotene and vitamin A in children is uncertain. Our current knowledge of the human metabolism of B-carotene is limited due to the inability to distinguish newly administered B-carotene and its metabolites from the body reserves of B-carotene and its metabolites namely retinol, retinoic acid, and retinyl esters. It is therefore of importance to study basic aspects of B-carotene absorption, metabolism, and its vitamin A equivalence using isotopically-labeled B-carotene. Tang et al. has developed mass spectrometry methods to quantify deuterated B-carotene and retinol in biological samples [7]. Using stable isotope labeled B-carotene and vitamin A in a childhood population in a geographic area with vitamin A deficiency, we will evaluate B-carotene bioavailability, body vitamin A storage, and bioconversion of B-carotene to retinol.

2. METHODS

2.1. Scientific scope of the project, detailed work plan for first year, including proposed methods or techniques

2.1.1. Preliminary study

2-3 mg retinyl acetate-d4 will be given to two administered non-infected child patients, then collect blood samples at 0, 6, and 24h, and at 2, 4, 6, 9, 15, 21, (25), and 35 days.

2.1.2. Study site and experimental design

The study will be conducted in Yuanyang County, Henan Province, situated in the southern part of Beijing, where it has been identified as a geographic area with vitamin A deficiency in childhood population. We shall cooperate with the Yuanyang County and Xinxiang City Anti-epidemic Stations. The study was approved by the Human Subjects Committee at the Chinese Academy of Preventive Medicine. Informed consent will be obtained from the parents or guardian of the participating children before enrollment in the study.

2.1.3. A screening investigation

A total of 300 pre-school kids (4-6 years old) in kindergarten will be studied. Anthropometric measurements (height and weight) will be recorded, in which, body weight will be measured with a digital scale, accurate to the nearest 20 g and length of the children will be measured to the nearest 0.1cm. Parasite infestation status in these children will be checked and a deworming pill will be given to those with positive test results. Demographic data will be collected, including family size, parents education, social economic and smoking status. Dietary history and 24-hour food consumption will be required in order to estimate carotene and vitamin A intakes. A total of 48 pre-school children with low vitamin A intake
in one kindergarten will be recruited to the study. About 24 of them in one kindergarten will be randomly assigned as an experimental group and other 24 will be assigned as a control group.

2.1.4. Intervention study

Vitamin A liver store of these children will be evaluated using 3 mg of retinyl acetate-d4. Forty-eight subjects under 7 years old, in the beginning of the experiment, will consume a 3 mg dose retinyl acetate-d4 in corn oil with a meal. The stable isotope dose will be accurately weighed (0.0001 g) and analyzed by HPLC analysis to confirm the contents. Serum samples (2.0 ml) will be taken at 14 and 25 days post dosing (fasting blood), the actual days taken samples will depend on the preliminary results. If the subjects vomit or the dose is regurgitated just after its administration the subject will not continue the study. To ensure that there are at least 48 subjects, 60 stable isotope doses will be included in this study.

2.1.5. The supplementation of food rich in pro-vitamin A carotenoids

In order to complete the food intake, the 48 subjects will have a daily meal in kindergarten, which will be rich or not rich in pro-vitamin A carotenoids. Fat intakes for subjects will be ensured in 20-25% of energy. Food intakes will be weighed using a digital scale for 3 months. During the experiment, 4.5 mg carotene for the subjects will be provided daily around Chinese RDA, the carotene intakes for control group will be around 2.1 mg/day (this is a general number but the actual intake in that area is probably below 2.1 mg/day). The Chinese RDA for children, and carotene content in common Chinese foods are shown in Table II and III. Recipes for supplying carotene are shown in Table IV. Food samples for each day will be collected to make homogenate and stored in -20°C for measuring carotene and vitamin A contents.

The effect of food based intervention on vitamin A status in children will be evaluated using 3 mg of retinyl acetate-d8. After two days stopping from the food supplementation for 3 months, the subjects will consume a 3 mg dose of retinyl acetate-d8 with a meal as in the beginning, then 2 serum samples (2.0 ml) will be taken at 14 and 25 days (fasting blood), the actual days taken samples will depend on the preliminary results. The blood samples will be sent to USA. The samples will be analyzed using mass spectrometry at Tufts University. Blood levels of retinoids, carotenoids, albumin, and zinc will be evaluated.

Statistics data entry will be performed in the field using an IBM-compatible PC with dBASEII. Means, standard deviation and standard error of the means will be calculated for all parameters.

3. RESULTS

The power of the study is estimated based on a survey carried in 1992, which showed that in 125 children (1-5 years), the prevalence of serum vitamin A levels below 0.70 μmol/L was 35.7% and the prevalence of serum vitamin A levels below 0.35 μmol/L was 11.1% in this area. The insufficiency of vitamin A was found more frequently in Yuanyang County, Henan Province. We expect to have 6-7 subjects, who are vitamin A deficient, in each group. We will then be able to evaluate the conversion efficiency of carotene to hepatic vitamin A in children with vitamin A deficiency or marginal vitamin A deficiency.
4. PLANS FOR FUTURE WORKS

4. 1. March - August 1996

- Establish the study station with necessary facilities, equipment, and supplies to collect, process, and store blood samples immediately and to distribute food supplements;
- Undertake a preliminary study to investigate retinyl acetate-d4 blood response curves in two administered children and to decide the most appropriate days to collect blood samples from the study groups.

4.2. September 1996

- 300 pre-school children (age 4-6 years) will be screened from parasites and be dewormed;
- Anthropometric measurements (height and weight) will be recorded;
- Dietary data and demographic data will be recorded. (Evaluation of liver storage of vitamin A using 3mg of retinyl acetate-d4.)

4.3. October - December 1996

Autumn is the best season to start the study because the foods supplied by us will be a better source in pro-vitamin A carotenoids than their daily diet. The supplementation of food rich in pro-vitamin A carotenoid will be carried for 10 weeks.


Evaluating the effect of food based intervention on vitamin A status in children using 3 mg of retinyl acetate-d8.

REFERENCES

### TABLE I. FOOD CONSUMPTION OF CHINESE IN 1982 AND 1992 (µg/man, day)

<table>
<thead>
<tr>
<th></th>
<th>1992</th>
<th>1982</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cereals</td>
<td>439.0</td>
<td>498.0</td>
</tr>
<tr>
<td>Dry beans</td>
<td>3.3</td>
<td>9.6</td>
</tr>
<tr>
<td>Bean products</td>
<td>8.0</td>
<td>5.3</td>
</tr>
<tr>
<td>Tubers</td>
<td>86.7</td>
<td>163.0</td>
</tr>
<tr>
<td>Pork</td>
<td>37.4</td>
<td>42.3</td>
</tr>
<tr>
<td>Eggs</td>
<td>16.1</td>
<td>9.7</td>
</tr>
<tr>
<td>Fish and shrimp</td>
<td>27.7</td>
<td>11.8</td>
</tr>
<tr>
<td>Milk</td>
<td>14.2</td>
<td>8.4</td>
</tr>
<tr>
<td>Deep color vegetables</td>
<td>102.9</td>
<td>74.0</td>
</tr>
<tr>
<td>Light color vegetables</td>
<td>209.5</td>
<td>224.0</td>
</tr>
<tr>
<td>Fresh fruits</td>
<td>49.7</td>
<td>28.0</td>
</tr>
<tr>
<td>Dry fruit</td>
<td>3.1</td>
<td>1.9</td>
</tr>
<tr>
<td>Sugars, starch</td>
<td>4.6</td>
<td>8.7</td>
</tr>
<tr>
<td>Vegetable oil</td>
<td>22.4</td>
<td>12.0</td>
</tr>
<tr>
<td>Animal oil</td>
<td>6.8</td>
<td>6.0</td>
</tr>
<tr>
<td>Salt</td>
<td>13.6</td>
<td>11.2</td>
</tr>
<tr>
<td>Soy sauce</td>
<td>12.7</td>
<td>12.8</td>
</tr>
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</table>

### TABLE II. RECOMMENDED DAILY ALLOWANCE FOR RETINOL

(Chinese Nutrition Society, 1989)

<table>
<thead>
<tr>
<th></th>
<th>0-12m</th>
<th>1 year</th>
<th>2 year</th>
<th>2-3 years</th>
<th>4-12 years</th>
</tr>
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<tbody>
<tr>
<td>Retinol Eqv. µg</td>
<td>200</td>
<td>300</td>
<td>400</td>
<td>500</td>
<td>750</td>
</tr>
</tbody>
</table>

### TABLE III. CAROTENE CONTENTS IN COMMON FOODS FROM CHINESE FOOD COMPOSITION TABLE (1991) (µg%)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>millet</td>
<td>100</td>
<td>17</td>
<td>sweet potato (red)</td>
<td>750</td>
<td>125</td>
</tr>
<tr>
<td>corn (yellow)</td>
<td>100</td>
<td>17</td>
<td>sweet potato (white)</td>
<td>220</td>
<td>37</td>
</tr>
<tr>
<td>corn flour (yellow)</td>
<td>40</td>
<td>7</td>
<td>carrot (red)</td>
<td>4130</td>
<td>688</td>
</tr>
<tr>
<td>broad bean (peeled)</td>
<td>300</td>
<td>50</td>
<td>carrot (yellow)</td>
<td>4010</td>
<td>668</td>
</tr>
<tr>
<td>bean jelly (dessert)</td>
<td>250</td>
<td>42</td>
<td>carrot (dehydrated)</td>
<td>17250</td>
<td>2875</td>
</tr>
<tr>
<td>soy-bean milk</td>
<td>90</td>
<td>15</td>
<td>ginger</td>
<td>170</td>
<td>28</td>
</tr>
<tr>
<td>fermented bean curd</td>
<td>130</td>
<td>22</td>
<td>radish</td>
<td>250</td>
<td>42</td>
</tr>
<tr>
<td>soybean</td>
<td>220</td>
<td>37</td>
<td>spinach</td>
<td>2920</td>
<td>487</td>
</tr>
<tr>
<td>soybean powder</td>
<td>380</td>
<td>63</td>
<td>spinach (dehydrated)</td>
<td>3590</td>
<td>598</td>
</tr>
<tr>
<td>green soya bean</td>
<td>790</td>
<td>132</td>
<td>fennel</td>
<td>2410</td>
<td>402</td>
</tr>
<tr>
<td>fresh kidney beans</td>
<td>220</td>
<td>37</td>
<td>Chinese chives</td>
<td>1410</td>
<td>235</td>
</tr>
<tr>
<td>tomato</td>
<td>550</td>
<td>92</td>
<td>peppery (red &amp; small)</td>
<td>1390</td>
<td>232</td>
</tr>
<tr>
<td>mushroom (dry)</td>
<td>1640</td>
<td>273</td>
<td>tangerine</td>
<td>180°5140</td>
<td>30°857</td>
</tr>
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### TABLE IV. RECIPES FOR SUPPLYING CAROTENE

<table>
<thead>
<tr>
<th>Week</th>
<th>Food</th>
<th>Experimental group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Consume (µg)</td>
<td>Carotene (µg)</td>
<td>Consume (µg)</td>
</tr>
<tr>
<td>1</td>
<td>Carrot</td>
<td>50</td>
<td>2000</td>
</tr>
<tr>
<td></td>
<td>Fennel</td>
<td>100</td>
<td>2400</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>4410</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Spinach</td>
<td>70</td>
<td>2044</td>
</tr>
<tr>
<td></td>
<td>Tangerine</td>
<td>50</td>
<td>2500</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>4544</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Tomato</td>
<td>50</td>
<td>275</td>
</tr>
<tr>
<td></td>
<td>Chinese chives</td>
<td>30</td>
<td>723</td>
</tr>
<tr>
<td></td>
<td>Carrot</td>
<td>20</td>
<td>3450</td>
</tr>
<tr>
<td></td>
<td>(dehydrated product as a dessert)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>4448</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Fennel</td>
<td>90</td>
<td>2048</td>
</tr>
<tr>
<td></td>
<td>Tangerine</td>
<td>50</td>
<td>2500</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>4548</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Sweet potato</td>
<td>50</td>
<td>375</td>
</tr>
<tr>
<td></td>
<td>Green soya bean</td>
<td>40</td>
<td>316</td>
</tr>
<tr>
<td></td>
<td>Chinese chives</td>
<td>15</td>
<td>341</td>
</tr>
<tr>
<td></td>
<td>Carrot</td>
<td>20</td>
<td>3450</td>
</tr>
<tr>
<td></td>
<td>(dehydrated product as a dessert)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>4502</td>
<td></td>
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</table>
DETERMINANTS OF VITAMIN A AND IRON STATUS IN PREGNANT MOTHERS AND PRESCHOOL CHILDREN

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Abstract

Micronutrient deficiencies, particularly deficiency of vitamin A and iron, have been recognized as a major public health problem in developing countries. This is particularly true for mothers, infants and preschool children, since these deficiencies may promote susceptibility to infectious agents and also increase the risk of developing chronic diseases in adult life. Increasing the availability of dietary provitamin A carotenoids from natural sources will improve vitamin A status among deficient populations. In order to evaluate such intervention programmes, it is essential to have biochemical and field assessment techniques. This proposal aims at conducting a demonstration project in Kerala, India using deuterated P-carotene as an intrinsic label to determine the utilization of this nutrient as a source of vitamin A. The programme design consists of i) method development phase during which Spirulina fusiformis will be grown in microreactors in presence of deuterated water for the production of labelled β-carotene and ii) an application phase wherein experimental animals (primates) will be administered labelled Spirulina to study the factors that influence the utilization of β-carotene for the formation of vitamin A.

1. SCIENTIFIC BACKGROUND AND SCOPE OF THE PROJECT

1.1. Introduction

This proposal describes a research project with the objective to assess the bioavailability and utilization of β-carotene as a source of vitamin A using biosynthetically labelled (deuterated) β-carotene as an intrinsic label. Deuterated Spirulina fusiformis will serve as the source of β-carotene. This study, proposed as part of the IAEA Coordinated Research Programme on Development and Application of Isotopic Techniques in studies on Vitamin A Nutrition, will provide scientific ground work for developing techniques for evaluation of dietary intervention programmes aimed at preventing micronutrient deficiencies and malnutrition and for promoting child survival particularly in areas where micronutrient deficiencies exist.

1.2. Rationale

Micronutrients have an important role in ensuring the health, development and survival of infants and children. Studies carried out in several countries suggest that deficiencies of vitamin A and iron have serious consequences [1-3]. Vitamin A is an
important micronutrient for sight, growth and reproduction. Vitamin A also has a critical role in cell differentiation and disease resistance. Vitamin A deficiency is a major problem affecting over 90 million children worldwide. Results of surveys from several Asian countries such as Indonesia, Philippines, India, Bangladesh, Nepal and Thailand [4-9] reveal that vitamin A deficiency is a problem in all or part of these countries. Increasing the availability of dietary provitamin A carotenoids will improve vitamin A status among deficient populations and such intervention may be of benefit to those with frank xerophthalmia as well as those children with subclinical state of vitamin A nutriture. Reports on the effect of vitamin A supplementation on childhood mortality also suggested that improving vitamin A nutrition should also be considered as one of the child survival strategies.

The major factors influencing the status of these micronutrients in individuals are availability of dietary sources, and adequacy of intake and absorption. Provitamin A carotenoid rich fruits and vegetables are important sources of dietary vitamin A. Such foods tend to be seasonal making it necessary to have intakes many times the normal daily requirement so as to ensure adequate liver stores of vitamin A. However, iron is stored in only limited amounts in the body, thus it must be ingested frequently, if deficiency is to be prevented.

Various strategies adopted to eradicate vitamin A and iron deficiency worldwide include tablet or capsule supplementation, food fortification and education to promote consumption of micronutrient rich foods. Dietary intervention through promoting the consumption of micronutrient rich food, though not very simple and quick, appears to be a sustainable public health measure for making durable improvements in vitamin A and iron status, for all members of the society. Dietary intervention to improve vitamin A status is all the more important in the special target groups which include pregnant and lactating women, infants and preschool children. A number of factors may affect the availability of vitamin A. These include availability of provitamin A in the diet, their conversion to vitamin A, rate of absorption, transport and the storage pool size. The dietary level of other nutrients such as fat, intestinal parasitism, frequent diarrhoeal diseases, etc. are some of the factors which also can affect the bioavailability of vitamin A.

In order to evaluate the dietary intervention programmes, it is essential to have reliable biochemical and field assessment techniques. Determination of plasma retinol levels may not give a true picture of the vitamin A status, because it is 'buffered'. More than 90% of the body vitamin A is stored in the liver and the hepatic vitamin A concentration is the best indicator of vitamin A status [13]. The relative dose response (RDR) and modified relative dose response (MRDR) are indirect tests used to identify subjects with low hepatic stores of vitamin A. These indirect methods may provide only an estimate of the total body stores of vitamin A which are needed to evaluate the extent of vitamin A deficiency. Isotope dilution analysis, as developed in studies using both \(^{1}\text{H}\) and \(^{2}\text{H}\) labelled vitamin A in a variety of species including human subjects, could be used for the calculation of hepatic vitamin A stores from the measurement of specific activity of plasma vitamin A [14]. Correlation between values obtained in direct measurement of vitamin A in hepatic biopsies and calculated values from isotope dilution analysis has been found [15]. The isotope dilution technique relies on the fact that following administration of a deuterated tracer dose and equilibration of the dose with the body vitamin A, the ratio of labelled to unlabelled retinol can be used to calculate the pool size by tracer dilution principles.

Of the different carotenoid pigments in plants, only a few can be cleaved to yield retinaldehyde. These include beta, alpha and gamma carotene and cryptoxanthin. \(\beta\)-carotene has the highest provitamin A activity. In developing countries approximately 80% of the vitamin A is derived from these carotenoids. This research project aims at developing intrinsically labelled (deuterated) carotenoids so that the absorption,
bioavailability and bioconversion of the provitamin A carotenoids of dietary origin can be studied.

1.3. Spirulina as a source of carotenoids

*Spirulina* is a microscopic plant, a form of algae that grows naturally in fresh water. It has a very high nutritive value owing to the presence of high content of proteins with all the essential amino acids, a blend of vitamins including B₁, B₂, B₆, B₁₂, C, E and biotin and trace elements like magnesium, zinc, potassium, etc. Apart from important pigments like chlorophyll, xanthophyll and phycocyanin, *Spirulina* has a carotene content 20 times that of carrot and iron content 12 times that of any other food and is the richest vegetarian source of vitamin B₁₂. The WHO has found *Spirulina* to be an excellent food for human consumption and it is being sold as a natural food. *Spirulina* has been easily incorporated in medical formulations owing to its excellent natural composition [16].

There are different varieties of *Spirulina* with varying contents of carotenoids. Further, storage and processing conditions also can affect the carotene content. In *Spirulina platensis* and *Spirulina fusiformis* total carotenoid content is about 4 mg/g of which β-carotene is about 1.7 - 2 mg. It is proposed to use *Spirulina* to prepare biosynthetically labelled deuterated provitamin A carotenoids. *Spirulina* containing intrinsically labelled provitamin A carotenoids can be used to study the influence of various factors on their absorption, bioavailability and bioconversion.

2. METHODS

2.1. Establishing *spirulina* cultures on agar

In order to prepare contaminant-free algal culture, *Spirulina fusiformis* available as stock culture will be inoculated on 2% agar-agar nutrient medium in a test tube. One to two drops of the stock liquid culture of *Spirulina* is added and spread uniformly, so that the growth is fast and uniform. The slants are kept at room temperature (25 - 30°C) in diffuse light (2-3K lux) subculturing is done in 20/30 days so that clean cultures are formed, and growth kinetics of algal cultures in suspension is studied by measuring the absorbence at 550 nm and by quantitation of biomass.

2.2. Designing a bioreactor for the production of deuterated *Spirulina fusiformis*

The methodology phase of this project proposal involves the design of a reactor for the production of deuterated *Spirulina fusiformis*. For this, suspension cultures will be grown in liquid medium prepared in deuterated water. Scrapings from clear agar slants are added to nutrient medium and kept under diffuse light (8-10k lux), at room temperature. Logarithmic phase cultures (as measured by absorbence at 550 nm) will be transferred to liquid medium (D₂O) in cylindrical tubes fitted into a chamber with light and temperature controls. Air mixed with CO₂ will be passed at regular intervals and are shaken intermittently to avoid settling. Stationary phase cultures will be collected, filtered and the biomass is collected. β-carotene is isolated and its content per gram of dry *Spirulina* will be determined. The isotope content in the β-carotene will be determined by mass spectrometric analysis.

3. RESULTS

Since the project has not started formally, only certain data relating to the standardization of the culture and the data related to the CRP generated by cooperating
institutions and scientists are presented. Since the commercially available cultures of *Spirulina* are meant for mass production, they are quite often contaminated with microbes and are not suitable for laboratory level production. Therefore attempts are being made to develop clean cultures by maintaining them on agar-agar and repeated subculturing.

As the algal cultures grow in suspension, the light absorbing particles increase. Therefore, the measurement of absorbence at 550 nm is used as a useful index to monitor the growth of the culture. The kinetics of growth of *Spirulina fusiformis* is followed to establish optimum conditions for biomass production and carotenoid enrichment.

The dried *Spirulina fusiformis* appeared to contain 55% protein, 15% carbohydrates, 9% ash, 10% crude fiber and about 5% fat. It has been demonstrated to be an effective dietary source of vitamin A. Studies carried out among preschool children with vitamin A deficiency has demonstrated that the bioavailability of carotenoids from *Spirulina fusiformis* is comparable to that from other sources, such as carrots and green leafy vegetables, suggesting the potential use of *Spirulina fusiformis* as a dietary source of vitamin A [17]. Evaluation of the chemopreventive potential of *Spirulina fusiformis* in reversing oral leukoplakia in pan tobacco chewers in Kerala by scientists at the Regional Cancer Centre, Trivandrum and U S D A, Beltsville, showed a complete regression of lesions in about 45% of valuable subjects receiving *Spirulina fusiformis* (1 g/day x 12 months) as opposed to 7% in the placebo arm [18]. In an extension of these studies, we conducted a double blind study of either vitamin A alone or β-carotene alone in subjects with oral leukoplakia in Kerala. The results based on a 12-month study on 43 compliant subjects on placebo, 42 on vitamin A, and 46 on β-carotene showed complete regression of 10% in the placebo arm, 52% with vitamin A and 33% with β-carotene [19]. This also points to the importance of bioavailability of vitamin A.

A major collaborative programme is underway in Trivandrum, India to determine the influence of intestinal parasitism on vitamin A and iron status in preschool children. This programme conducted in collaboration with the Johns Hopkins University, Department of International Health and USDA, has developed appropriate instruments for the measurement of nutrient intake.

4. PLANS FOR FUTURE WORK

During the first year of the programme, which is the methods development phase, *Spirulina fusiformis* will be grown in micro reactors in the presence of deuterated water for the production of intrinsically labelled β-carotene. In the second phase, which is the application phase, experimental animals (primates) will be administered labelled *Spirulina* to study the factors that influence the utilization of β-carotene. The labelled β-carotene produced can be used for studies on absorption, bioavailability and conversion into vitamin A by other centres involved in this CRP.
REFERENCES


AN IMPROVED VITAMIN A CARRIER FOR CHILDREN 6 MONTHS-3 YEARS OF AGE

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Abstract

Vitamin A deficiency (VAD), is strongly linked to blindness in addition to presenting an increased risk of death and morbidity from gastrointestinal and respiratory diseases. An edible sponge, fulfilling the criteria as a viable micronutrient carrier, would answer and facilitate the delivery of a logical control to this situation. The authors propose creation of a new technology to be carried out in the Institute of Biochemistry, Faculty of Agriculture of The Hebrew University of Jerusalem. This novel and innovative approach will focus on hydrocolloid matrices to which oil has been added by homogenization followed by a gelation process. The ensuing gel will be freeze-dried to yield a crunchy chewable cellular solid (edible sponge), designed as packaging for vitamin A and minerals. The product will be studied for mechanical (textural) properties to allow customized, affordable stable packaging for minerals and vitamins together. In parallel, the two research teams will examine sponge digestibility, stability and efficiency through intake experiments on animals and children. The advantages of the proposed technology include containment of water and oil-soluble ingredients in one package, ease of production, low price and availability of raw materials in addition to the possible adaptability for different age groups. Since the sponge is fibrous, it is void of flavor, odor, and color. Hence, it is possible to control and incorporate these characteristics during processing, thus ensuring broad acceptance by the targeted subjects. The proposed methodology provides a fortified, stable and affordable food product successfully comprising more than one micronutrient.

1. SCIENTIFIC BACKGROUND AND SCOPE OF THE PROJECT

The relationship between inadequate vitamin A status and xerophthalmia (nutritional blindness) has been known for centuries. The importance of vitamin A to ocular integrity and resistance to infections is extensively documented [1]. Vitamin A is an essential component of the rhodopsin cycle, the visual pigment required for vision under low levels of illumination [2]; thus, early deficiency shows a night blindness. Ocular manifestations associated with vitamin A deficiency are reversible during the early stages with supplementation or consumption of adequate intake of vitamin A without significant sequelae except corneal ulceration with resultant scaring. Research on animals and humans has revealed the role of vitamin A in the etiology of iron deficiency anemia. A recent study of pregnant Indonesian women indicated greater effectiveness of a combined iron supplement and vitamin A (97%) in reducing anemia than iron tablets alone (68%) and 16% in the control group receiving a placebo [3]. At present, three approaches address the problem of VAD: (i) a bi-annual distribution of a high dose of vitamin A to preschool children in areas where VAD is of public health importance; (ii) horticulture and nutrition education and; (iii) food fortification. However, at this point, they are technologically possible only in the experimental phase in a number of countries due to costs and the difficulty in selecting a vehicle and a food item regularly eaten by the target group in sufficient quantities [4]. The general objective of the proposed project is improvement of the nutritional status of poor communities in Ethiopia through food-fortification, with particular
reference to vitamin A. Development of a technology to produce an affordable, stable, and
efficacious vitamin A fortified edible hydrocolloid sponge can meet this challenge. Phase
I of the program will center on construction of a sponge incorporating therapeutic amounts
of iron and vitamin A. Phase II will include: (i) sponge-product acceptability (color, taste,
texture) testing on a selected group of Ethiopian infants and young toddlers given the
vitamin A fortified sponge; (ii) animal studies to examine the availability and status of iron
and vitamin A in rats fed diets supplemented with the fortified sponge. Iron and vitamin A
levels will be monitored weekly for 6 weeks using high-pressure liquid chromatography
(HPLC) for detection of vitamin A level and inductively coupled plasma (ICP) for detection
of iron as well as Ferritin, an iron marker, which will be tested by a commercially available
kit; (iii) the production of the sponge will be transferred to Ethiopia prior to the field study
in Gondar, Ethiopia. Infants identified as vitamin A deficient will be provided with the
sponge. Iron and vitamin A levels will be monitored and acceptability and palatability tests
will be performed. Phase III will evaluate the process related to production, use and
consumption of the fortified sponge with special emphasis on vitamin A and iron status. The
results will delineate the potential and constraints in the attempt to fortify vitamin A and
iron micro-nutrients in an edible sponge. At this stage a stable isotope dilution technique
will be introduced to assess the vitamin A status in a selected group of children receiving
the sponge.

2. METHODS

2.1. Gum-oil gels and sponges

Food-grade commercial alginate will be used to prepare the gels using a procedure
previously described [5,6]. The alginate will carry the water soluble micronutrients designed
to be included within the matrix. Low quantities of soya oil (as a medium for oil soluble
ingredients) will be added by homogenization followed by glucono-δ-lactone (GDL) solution
as a gelation inducer. Emulsifier will be included in the oil before homogenization as well
as antioxidants to retard or prevent oxidation of vitamins and oil during the shelf life of the
designed product. Gels will be kept at 5°C for 24 hours, later compressed to check their
mechanical properties between parallel lubricated plates at a constant deformation
(displacement) rate of 10 mm/min, using an Instron universal testing machine Model 1100.
All mechanical tests will be performed in triplicate, with samples taken from two separate
batches. These tests can serve to control the textural properties of the future products.
Then, the matrices will be dried and compressed as sponges, as described below. Drying
is designed to ensure extended shelf life, stability, crunchiness and to inhibit rate of
oxidation rate.

2.2. Mechanical properties and porosity of products (edible sponges)

The different sponges will be compressed to 80% deformation between parallel
lubricated plates at a constant deformation (displacement) rate of 10 mm/min, using the
Instron. Texture studies on the sponges is crucial for future texture control of the products.
The Instron’s continuous voltage vs. time output will be converted into stress vs. Hencky’s
(natural) strain relationships [1,2]:

\[ \sigma = \frac{F}{AO} \quad \text{and} \quad \varepsilon_h = \ln \left[ \frac{H_0}{H(t) - \Delta H} \right] \]

where \( \sigma \) and \( \varepsilon \) are stress and Hencky’s strain, respectively, \( F \) is the momentary force, \( \Delta H = H_0 - H(t) \) is the momentary deformation, \( AO \) and \( H_0 \) are the original specimen’s cross
sectional area and height, respectively, and \( H(t) \) is the height at time \( t \). Since the
cross-sectional area of a compressed solid sponge specimen rarely expands to any
significant extent [7], the engineering and "true" stresses could be treated as equal for all
practical purposes [8].
Additional information on the influence of added ingredients and different conditions, i.e., technology of drying, temperatures, times, content of oil, etc., affecting the texture of the final products, will be gathered by fitting the individual relationships to a compressibility model previously developed for the sigmoid stress-strain relationships of cellular solids [9,10]:

\[ \sigma = \frac{C_1}{(1 + C_2 e C_3 e)} \]

where \( \sigma \) and \( e \) are the stress and strain, respectively, and \( C_1 \), \( C_2 \) and \( C_3 \) are constants calculated by non-linear regression of the Systat package. Since porosity of the sponges is important to their textural properties and crunchiness it will be expressed as total porosity \( = 1 - (\text{bulk density} / \text{solid density}) \) [10].

2.3. Scanning electron microscopy (SEM)

SEM micrographs of sponges will be studied to check their structure which is related to their success as products.

The use of stable isotope tracers of vitamin A will be used to assess the vitamin A status prior and after the introduction of the sponge to the vitamin A deficient children. The specific methodology will be determined with the experts of the IAEA after the first field study in Gondar.

2.4. Time schedule

The study is planned for 24-30 months.

Phase I 8-10 months
Phase II 8-10 months
Phase III 8-10 months

REFERENCES

APPLICATION OF THE DEUTERATED RETINOL DILUTION TECHNIQUE TO DETECT CHANGES IN HEPATIC RETINOL RESERVES IN INFANTS WHO RECEIVE HIGH DOSE VITAMIN A SUPPLEMENTS

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Abstract

The purpose of this pilot study is to determine whether the deuterated retinol dilution technique can detect changes in hepatic vitamin A reserves in Peruvian infants in response to supplementation with vitamin A palmitate. Approximately 40 infants, 9 months of age, will be recruited for the study and randomly assigned to four treatment groups. Hepatic vitamin A reserves will be estimated using the deuterated retinol dilution technique before and after administration of a single supplement containing either 0, 7.5, 15, or 30 mg RE as retinyl palmitate in corn oil. Mean hepatic vitamin A reserves will be calculated based on the plasma isotopic ratio of d4-retinol:retinol as per Furr et al. [1]. Hepatic vitamin A reserves will be compared within groups before and after supplementation, and among the four groups. The mean change in hepatic vitamin A reserves across the four treatment groups will be examined in relation to the amount of vitamin A supplement administered, using regression analysis. The results of the pilot study will provide information on the size and variation of hepatic vitamin A reserves in 9-month old Peruvian infants. This information will be useful for calculating sample sizes to evaluate vitamin A intervention strategies by estimating changes in hepatic vitamin A stores in this age group, using the DRD technique.

1. SCIENTIFIC BACKGROUND AND SCOPE OF THE PROJECT

Measuring vitamin A status in human subjects is problematic because commonly used assessment techniques do not provide a quantitative estimate of body stores of vitamin A. Serum retinol concentrations are maintained within a narrow range, and only change at the extremes of vitamin A depletion or toxicity [2]. The relative dose response tests classify individuals as depleted or normal only, and do not provide a quantitative estimate of body stores. In well-nourished individuals, it is estimated that greater than 90% of total body vitamin A is stored in the liver as retinyl esters [3]. For this reason, the hepatic concentration of vitamin A is considered to be the best indicator of vitamin A status. Obviously, it is not feasible to obtain liver biopsy specimens from healthy individuals for direct assessment of their vitamin A status. However, the deuterated retinol dilution (DRD) technique was recently introduced as an indirect method for quantitatively estimating hepatic reserves of vitamin A across the full continuum of vitamin A status. The isotope dilution technique was validated in rats [4], and partially validated in generally healthy human surgical patients in the U.S. [1]. Briefly, a known dose of retinyl-d4 acetate is administered orally, and after a period of equilibration with the body pool of vitamin A, a blood sample is drawn for measurement of the isotopic ratio of retinol-d4/retinol in plasma. Hepatic reserves of vitamin A can be calculated based on the principles of isotope dilution, and a set of assumptions, first described by Bausch and Reitz [4] and later modified by Furr et al. [1]. The technique was partially validated in eleven well-nourished surgical patients in the United States, by comparing hepatic retinol concentrations estimated by the isotope dilution technique with direct measurement of the hepatic retinol concentration in liver biopsy specimens obtained at the time of surgery [1]. When one subject was omitted from
the analysis, a good correlation was found between the two techniques \((r = 0.88)\). However, only one of the eleven patients had a low \((<70 \text{ nmol/g})\) hepatic retinol concentration. Thus, it was not clear if the DRD technique, as described by Furr et. al. [1], could provide a quantitative estimate of hepatic vitamin A reserves in a population with low vitamin A body stores.

We have recently conducted a study in Bangladesh to examine the relationship between DRD technique and hepatic retinol reserves in a population with low body stores of vitamin A [5]. Briefly, hepatic vitamin A reserves were estimated by the DRD technique and by direct measurement of the retinol concentration of a liver biopsy specimen that was obtained at the time of surgery in 31 adult surgical patients in Bangladesh who had been previously scheduled for surgery for peptic ulcer or gall bladder disease. Nearly half \((n = 14, 45\%)\) of these patients had hepatic retinol concentrations \(<70 \text{ nmol/g}\), based on direct measurement of the retinol concentration of the biopsy specimen. A significant linear relationship was found between hepatic vitamin A reserves, as estimated by the DRD technique or by direct measurement of retinol concentration of the biopsy specimen \((r = 0.69, p<0.0001)\). Agreement between the two techniques was poor in several subjects, three of whom had clinical conditions consistent with poor absorption or retention of the dose of retinyl-d4-acetate. When these three subjects are omitted from the analysis, the relationship between the two techniques is improved \((r = 0.83)\). Thus, the DRD technique, as described by Furr et. al. [1], provided a reasonably good quantitative estimate of hepatic retinol A reserves in this population of surgical patients with low to adequate vitamin A body stores.

A second study was conducted in Bangladesh to assess the responsiveness of the DRD technique to known differences in vitamin A intake in a group of adult volunteers [6]. Briefly, plasma isotopic ratios of d4-retinol:retinol were measured at the beginning and end of 90-d periods of supervised consumption of different levels of vitamin A in 30 adult, male volunteers. Each subject received a standardized diet, containing very low levels of vitamin A throughout the 127-d observation period, as well as a daily capsule of either 0, 5.2, or 10.5 \(\times 10^6\) mol retinol as unlabelled retinyl palmitate in corn oil, in randomized, masked fashion, for 75 d. Oral doses of d4-retinyl acetate \((45.2 = 10^6\) mol) were given on days 1 and 90. Preliminary results of plasma isotopic ratios are available from 15 subjects on days 2, 30, 60, 90, and 127. The mean plasma isotopic ratios were similar initially. By day 30 and thereafter the ratios were significantly greater in the groups that consumed less vitamin A. Thus, plasma isotopic ratios respond within several weeks to differences in daily vitamin A intake, indicating that the DRD technique can detect differences in vitamin A consumption.

In countries where vitamin A deficiency is a significant public health problem, direct supplementation of young children with high-dose vitamin A supplements is currently recommended every 6 months, from 6 months to 6 years of age [7]. Administration of vitamin A to infants less than 6 months of age in conjunction with the Expanded Program of Immunization (EPI) has also been suggested as a strategy for reducing the incidence of vitamin A deficiency during the first 12-18 months of life. The rationale of early supplementation with vitamin A is to provide infants with adequate hepatic stores of vitamin A during 6 - 18 months of age when they are at increased risk of vitamin A deficiency due to common childhood diseases, and to a reduction in breast-milk intake and the introduction of complementary foods that are often low in vitamin A. Given the high coverage of the EPI program in many developing countries, administration of vitamin A at the EPI contacts may be an effective strategy for maintaining adequate vitamin A status during the first 12-18 months of life. Early supplementation, theoretically, should be beneficial to young infants, however, little information is available on the safety and efficacy of administration of high-dose supplements to infants in this age group.
The World Health Organization is currently conducting a multi-center trial to determine the efficacy and safety of early supplementation with vitamin A in conjunction with the EPI. The Instituto de Investigacion Nutricional in Lima, Peru has been chosen as one of the centers to participate in the evaluation of the WHO/EPI-linked vitamin A supplementation trial. Infants participating in the WHO/EPI trial will receive 25,000 IU of vitamin A at their first 3 EPI visits (2, 3 and 4 months of age), and a final supplement at their last EPI visit at 9 months of age. Infants in the control group will receive a placebo at their first 3 EPI contacts, but will receive a dose of 100,000 IU of vitamin A at their final EPI visit at 9 months of age. Commonly used assessment techniques, such as serum retinol concentration, and the modified relative dose response test will be used to detect changes in vitamin A status in the WHO/EPI trial. To evaluate the efficacy of this trial, the DRD technique could be used to quantitatively measure changes in vitamin A body stores in response to supplementation.

At 9 months of age, before they receive their final supplement, infants in the treatment group will have received 3 doses of vitamin A (75,000 IU in total). Presumably, supplemented children will have higher hepatic reserves of vitamin A than non-supplemented children, thus the efficacy of the WHO/EPI supplementation trial could be evaluated using the DRD technique to quantitatively compare hepatic reserves of vitamin A at 9 months of age in infants in the treatment and control groups.

Since the DRD technique has not yet been used to assess hepatic stores in infants, we are conducting a pilot study to assess the sensitivity of the DRD technique for detecting the expected increase in hepatic reserves of vitamin A in response to supplementation. The pilot study will provide information on i) the hepatic reserves of vitamin A in 9-month old Peruvian infants, and ii) the retention, or expected change in hepatic vitamin A reserves, in response to three different levels of high-dose vitamin A supplements. With this information, it will be possible to calculate the sample size that will be required for an evaluation of the EPI-linked supplementation trial, based on the variation in hepatic vitamin A reserves in this age group, and the variation in retention of vitamin A in response to supplementation.

2. METHODS

2.1. Objectives

a. To measure the hepatic reserves of vitamin A in infants 9 months of age, at baseline and again one month after they receive a single high-dose vitamin A supplement containing either 0 mg RE, 7.5 mg RE, 15 mg RE, or 30 mg RE, to estimate the increase in hepatic reserves of vitamin A in response to supplementation with vitamin A.

b. To describe the mean change in hepatic reserves across the four treatment groups in relation to the amount of vitamin A administered.

2.2. Hypotheses

a. The DRD technique will detect a significant increase in hepatic vitamin A reserves in children who receive a single high-dose supplement containing 7.5 mg RE, 15 mg RE, or 30 mg RE of vitamin A.

b. The mean change in hepatic vitamin A reserves will be different across the three groups that receive vitamin A supplements, and there will be a linear relationship between the mean change in hepatic vitamin A reserves and the dose of vitamin A administered.
2.3. Study design

Forty-seven infants, 9 months of age, have been recruited for the study and randomly assigned to 4 treatment groups. Prior to selection, they were examined by physician, and were considered eligible for the study if they were free from fever and infectious disease. At baseline, the infants were weighed and measured, a questionnaire on feeding practices was administered to the mother, and a maternal breast-milk sample was collected for determination of the retinol concentration. Upon completion of these assessments, an oral dose of 1.5 mg RE of d4-retinyl acetate was administered to the infants. At this point, all of the infants have received their first dose of isotope, however, blood samples have not yet been collected. A blood sample (3 mL) will be drawn 21 days post-dose (study day 21) for measurement of the isotopic ratio of retinol-d4/retinol in plasma to estimate hepatic vitamin A reserves at baseline. After the blood is drawn, an oral dose of either 0 mg RE (corn oil), 7.5 mg RE, 15 mg RE, or 30 mg RE of vitamin A will be administered to the infants as retinyl palmitate in corn oil. A second blood sample (3 ml) will be drawn 30 days later (study day 51) for measurement of the isotopic ratio of retinol-d4/retinol in plasma. Immediately thereafter, a second dose of d4-retinyl acetate will be administered orally to the infants. A third blood sample will be obtained 21 days later (study day 72), for measurement of the isotopic ratio of retinol-d4/retinol in plasma to estimate hepatic reserves after supplementation with a high-dose vitamin A supplement. A field worker has been visiting the infants in their homes weekly to monitor morbidity, and will continue to do so throughout the study period. Mothers have been instructed not to administer any vitamin A supplements to the infants while they are participating in the study.

2.4. Dose of d4-retinyl acetate and sampling time

The dose of d4-retinyl acetate to be administered to the infants was calculated based on the assumption that hepatic reserves of vitamin A in 9-month old infants is likely to range from 10 ug/g - 100 ug/g. The average weight of Peruvian children 6-11 months of age is 8.3 kg [8], thus liver weight can be estimated as ~200 g (2.4% of body weight). If the hepatic vitamin A concentration ranges from 10 - 100 ug/g, hepatic stores of vitamin A can be estimated to range from 2 - 20 mg. Assuming hepatic reserves represent ~90% of body stores of vitamin A, total body stores of vitamin A can be estimated to range from 2.2 - 22.2 mg. Assuming 50% of an oral dose of vitamin A is retained [3], a dose of 1.5 mg of vitamin A would achieve ~3.4 - 34% labelling of the body pool.

To estimate hepatic reserves of vitamin A in adults using the DRD technique, blood samples are drawn after the isotope has "equilibrated" with the body pool of vitamin A. In our preliminary studies of the kinetics of equilibration in two adults and one 6 year old child, we have estimated that equilibration occurs between 12 - 27 days after the isotope is administered (Figures 1-3) [9]. For this pilot study in young infants, we have chosen to draw blood 21 days after administering the isotope. It is necessary to draw blood prior to administering the second dose of d4-retinyl acetate to determine the slope of the post-equilibration plasma decay curve in order to estimate the contribution of retinol-d4 from the first dose, to the isotopic ratio of retinol-d4/retinol in plasma (study day 72) after the second dose of isotope is administered.

2.5. Data analysis

Descriptive statistics will be calculated for all variables. The difference in mean hepatic reserves pre- and post-supplementation within treatment groups will be compared using the paired test. The difference in the mean change in hepatic reserves across treatment groups will be compared by analysis of variance. The mean change in hepatic reserves of vitamin A in the four treatment groups will be evaluated in relation to the level of vitamin A supplementation using regression analysis.
2.6. Laboratory analyses

*Plasma retinol:* For determination of the plasma retinol concentration, an aliquot of plasma will be treated with ethanol to denature protein, and vitamin A will be extracted into hexane using retinyl acetate as an internal standard. The hexane layer will be separated by centrifugation, transferred to a clean vial and evaporated under N2. The residue will be dissolved in methanol, and the retinol concentration will be determined by isocratic reversed-phased HPLC [10]. To assess the accuracy and precision of the retinol measurements, control serum from the National Institute of Standards will be run periodically.

*Isotopic ratio of retinol-d4/retinol:* The plasma isotopic ratios of retinol-d4/retinol will be estimated by GC/MS according to the method reported by Clifford *et al.* [11], as modified by Handelman *et al.* [12].
3. RESULTS

At this point in time, the recruiting phase has been completed and 47 infants have received their initial doses of retinyl-d4 acetate in corn oil. Additional information will be reported as it becomes available.

4. PLANS FOR FUTURE WORK

We are planning to use the DRD technique to evaluate the WHO/EPI-linked vitamin A supplementation trial which is taking place in Lima, Peru. The results of the pilot study will provide us with information on the size and variation of hepatic vitamin A reserves of 9-month old infants in the neighborhoods where the WHO/EPI trial is taking place. This information will enable us to calculate the sample size required to detect a difference in the vitamin A stores of infants who do and do not participate in the WHO/EPI-linked trial. In addition to the work in Peru, we have recently conducted a pilot study in Ghana to estimate changes in hepatic reserves of vitamin A using the DRD technique in ~3-4-month old infants who received a single high-dose vitamin A supplement.

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USE OF STABLE ISOTOPES IN STUDIES OF VITAMIN A NUTRITION IN THE PHILIPPINES

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Abstract

Vitamin A deficiency is recognized as a public health problem in the Philippines as confirmed by the 1993 National Nutrition Survey which showed an increasing prevalence of vitamin A deficiency among schoolchildren. This proposed study aims to determine whether the intake of fruits and vegetables that are rich in provitamin A carotenoids will result in an improvement in vitamin A liver stores of 7-10 year old children who are malnourished in vitamin A. To meet this objective, vitamin A malnourished children (serum retinol, <25 µg/dL) in two villages (experimental and control) will be identified and enrolled. Deworming will be done one month before the start of the study. Vitamin A status measurements - the deuterated retinol dilution method, serum retinol, dietary vitamin A intake, and conjunctival impression cytology (CIC) procedure - will be done at baseline. Then the experimental group (n = 25) will be fed with fruits/vegetables to provide 2.4 mg of β-carotene, i.e., 400 µg retinol equivalents (RE) daily, for five days a week for twelve weeks. The control group (n = 25) will be fed an equivalent amount of calories and no vitamin A or carotenes. At the end of the feeding period, all measurements for determining vitamin A status will be repeated. Other determinations before and after intervention, will include total blood haemoglobin, and serum ferritin, albumin, transthyretin, and C-reactive protein concentrations. The proposed study will provide information regarding the adequacy of vitamin A measures, and the efficacy of food-based intervention programs in improving the vitamin A status of malnourished populations.

1. SCIENTIFIC BACKGROUND AND SCOPE OF THE PROJECT

1.1. Background

Vitamin A deficiency continues to be a public health problem in many developing countries including the Philippines. It is estimated that 17 children in the Philippines go blind everyday as a result of vitamin A deficiency [1]. As shown in Table I, vitamin A deficiency signs and symptoms were found in many regions of the country [2-7]. Among schoolchildren 7-14 years of age, 1.3% experienced night blindness, and 0.2% had Bitot's spots [7]. In certain regions, low serum retinol concentrations (< 20 µg/dL) were highly prevalent [2, 6, 8-11]. For example, a study done by the Nutrition Center of the Philippines in 1993 showed that about 30% of rural schoolchildren 6-16 years old, in Santo Tomas, Batangas, had serum retinol levels that were <20 µg/dL [11]. In rural areas, dietary vitamin A intake was only 73% of the Filipino recommended daily intake, and was obtained mostly from pro-vitamin A carotenoid sources.
1.2. Study Objectives

This study aims to determine whether the intake of fruits and vegetables that are rich in pro-vitamin A carotenoids, i.e., 2.4 mg β-carotene (400 µg RE), for 5 days a week for 12 weeks, will result in an improvement in the vitamin A status of 7-10 year old children who are malnourished in vitamin A. The results of the deuterated retinol dilution method will be compared with other measures of assessing vitamin A status, i.e., serum retinol concentrations, total dietary vitamin A intake from pre-formed vitamin A and pro-vitamin A carotenoids, and the CIC procedure.

1.3. Deuterated Retinol Dilution Method (DRD)

A most promising indirect approach for estimating total body stores of vitamin A appears to be the stable isotope dilution method using deuterated vitamin A [12-15]. The method is based on the principle that when a tracer dose of deuterated vitamin A is administered, it reaches a pseudo equilibrium with the existing body pool of vitamin A, and the ratio of deuterated to non-deuterated retinol in serum can be used to calculate total body stores, 90-95% of which is in the liver [16,17]. Since deuterated vitamin A is non-radioactive, there are no risks involved by ingesting it. Earlier studies with animals employed tracer doses of radioactive (3H) vitamin A to validate the use of the isotope dilution method as a measure of total body stores of vitamin A [18-21]. Few studies have been done in humans. Sauberlich et al. used (C14) vitamin A to study the vitamin A body pool and utilization rate in young adult males [16]. Furr et al. validated the use of the isotope dilution assay using tetradeuterated vitamin A by comparing the calculated vitamin A liver concentrations with values obtained by HPLC measurements of surgical liver biopsies [12]. They found that the correlation coefficient between these values was 0.88, and the Spearman's rank correlation coefficient was 0.95 (p<0.002). They concluded that the isotope dilution assay can provide a valid estimate of total body stores of vitamin A in humans. As one might expect, Furr et al. found no correlation in the satisfactory range of vitamin A status, between the results of the stable isotope dilution method and serum retinol levels, since serum retinol is homoeostatically controlled over a wide normal range of liver vitamin A levels [12, 22].

1.4. Study Significance

Data from this study will provide information regarding the efficacy of food-based intervention programs in improving the vitamin A status of malnourished populations. It will indicate how the deuterated retinol dilution assay compares with other measures of assessing vitamin A status, i.e., total dietary vitamin A intake, serum retinol concentrations, and the CIC procedure. Correlations with total dietary intakes will show at which level of intake low body vitamin A stores (<20 µg/g liver) are associated with.

2. METHODS

2.1. Preliminary procedures

These include identification of the study sites which are two elementary schools located in two villages of similar socio-economic characteristics; conferring with local government, school and health officials; obtaining study approval from the Ethics Review Board of the Philippine Council for Health and Research Development; conferring with various agencies regarding food supplies; and setting up of a "work station" at the study sites.
2.2. Recruitment of subjects

Elementary school children of both sexes will be recruited to participate. Informed consent will be obtained from their parents or guardians.

2.3. Screening

2.3.1. Procedures and exclusions

Volunteer subjects (total n = 400 or 200 per school) will undergo screening procedures as follows: information will be collected on medical history, use of medications, nutritional supplements, age, parents' smoking habits, and demographic characteristics (e.g., family size, parents' education, etc.). Eye examinations will be conducted by an ophthalmologist, and children found to have clinical signs of xerophthalmia (Bitot's spots and conjunctival xerosis) will be treated and excluded from participation [22]. A complete physical examination will be done; heights and weights will be obtained. General nutritional state will be determined by use of Filipino standard tables of weight-for-age, and weight-for-height, and height-for-age measurements. Those who are <60% of weight-for-age gender-specific standards will be considered severely protein-energy malnourished, and will not be eligible to participate. The children who will be admitted into the study will be 7-10 years of age who do not have any major diseases, prolonged diarrhea, acute or chronic infections, and prolonged febrile conditions. They should agree to provide blood samples during the study, and should be permanent residents in the area.

2.3.2. Conjunctiva/ impression cytology (CIC)

As part of the screening procedure, in addition to an eye examination for signs of xerophthalmia, a cytologic assessment will be done. The CIC procedure is a non-invasive and sensitive histological technique of obtaining surface cells on the bulbar conjunctiva with the use of a special vacuum pump applicator that gently applies a filter paper on the temporal surface of each eye and removes it by suction in one quick step [23]. The specimen is fixed, stained and examined under light microscopy for the presence of abundant mucin-secreting goblet cells and sheets of small epithelial cells. Impressions from xerophthalmic children will show a marked reduction or absence of goblet cells and the appearance of enlarged keratinized epithelial cells [24].

2.3.3. Serum retinol measurements

To identify vitamin A-deficient children, blood will be obtained from 300 children (~150 per school). Serum will be separated, frozen, and transported to the HNRCA in Boston for serum retinol analyses using reverse-phase HPLC procedures (25,26). Those with serum retinol values of <25 µg/dL will be considered vitamin A-deficient and thus eligible to participate in the intervention study.

2.4. Study design

Children in the experimental group (n = 25) whose serum retinol values are found to be <25 µg/dL, will be matched (for age, gender, weight for age, family size, serum retinol value) to children in the control group (n = 25). The following procedures will be done: i) deworming; ii) baseline measurements of vitamin A status and other biochemical measurements in blood; iii) feeding fruits and vegetables during the intervention period to the experimental group, and an equivalent amount of calories from foods containing no vitamin A or carotenoids to the control group; iv) and at post-intervention, repeat of vitamin A status measurements and other blood measurements.
2.4.1. Deworming

About one month before the start of the intervention phase, the participants will be treated for parasites, since parasites may adversely affect carotenoid absorption and vitamin A status.

2.4.2. Baseline measurements

2.4.2.1. Deuterated retinol dilution test

Subjects will start an isotope dilution test by oral ingestion of 4 mg of tetradeuterated vitamin A (all-trans-retinyl-10, 19, 19, 19-[2H4]acetate) in corn oil. After a pseudo equilibrium period of 21 days, 5 mL of blood will be drawn; serum will be separated, frozen, and transported to the HNRCA in Boston for HPLC quantitation of retinol [25,26], isolation of retinol from serum, conversion of isolated retinol to tert-butyldimethylsilyl derivatives [14], and determination of deuterated and non-deuterated retinol in derivatized preparations by GC/MS [12-15]. Total body stores of vitamin A will be calculated using the formula of Bausch and Rietz as modified by Furr et al. [12, 18].

2.4.2.2. Dietary Vitamin A intake assessment

A detailed assessment of the child's dietary intakes of vitamin A, pro-vitamin A carotenoids, protein, fat, and energy, will be obtained by a dietitian. Retrospective methods for recalling past actual intake (e.g., 24-hour food recall) and for determining past usual intake through a food frequency questionnaire based on Philippine food composition tables will be used.

2.4.2.3. Other biochemical measurements in blood

In addition, the following measurements will be done. Serum carotenoids will be quantitated using HPLC procedures that measure both pro-vitamin A carotenoids (α- and β-carotene, cryptoxanthin) and non-pro-vitamin A carotenoids (lycopene, lutein, zeaxanthin) [25,26]. Total haemoglobin in whole blood, and serum ferritin, albumin, transthyretin, and C-reactive protein will be measured. Serum ferritin will be determined using the Ciba Corning MAGIC ferritin (125I) radioimmunoassay kit (Cat. No. 472329, Ciba Corning Diagnostics Corp., Medfield, MA). Serum albumin will be measured using the Roche Reagent for Albumin kit (Order No. 42332, Roche Diagnostic Systems, Branchburg, NJ) which uses a modification of the bromocresol green binding assay of Doumas et al. [27]. C-reactive protein will be measured with the Cobas FARA II in conjunction with a reagents kit from Atlantic Antibodies (Document AM-0039, Atlantic Antibodies, Inc., Stillwater, MN). Except for haemoglobin determinations, all of these assays will be conducted at the HNRCA in Boston.

2.4.3. Food-intervention phase: Effect of fruit and vegetable intake on measures of vitamin A status

Five days a week for twelve weeks, the experimental group will be fed with fruits/vegetables with rice, to provide 2.4 mg of β-carotene (i.e., 40 0 μg RE) per day; the control group will be fed an equivalent amount of calories in the form of cereal/rice products and jam devoid of vitamin A and carotenes. After the study period of 12 weeks, all measurements done at baseline, the CIC procedure, and serum retinol measurements will be repeated. For the isotope dilution test, octadeuterated retinyl acetate will be administered (instead of tetradeuterated retinyl acetate) in order to avoid interference from residual tetradeuterated retinol from the previous dose.
Throughout the intervention phase, morbidity data will be obtained, and weekly eye examinations will be done in both experimental and control groups. Subjects who develop clinical signs of xerophthalmia will be treated and removed from the study. At the end of the 12-week period, all children will be given a 200 000 IU dose of vitamin A before they are discharged from the study. (All schoolchildren in the two villages who are identified to have low serum retinol levels will be provided with a 200 000 IU vitamin A capsule upon completion of the study, and every 6 months thereafter, for a period of 2 years.)

Whether pro-vitamin A carotenoids from plants can improve vitamin A status in humans remains controversial [28]. A recent study by de Pee et al. showed no improvement in vitamin A status of breast feeding Indonesian women supplemented daily with 100-150 g of vegetables (cassava leaves, water spinach, spinach, or carrots) for 12 weeks, whereas a similar amount of β-carotene from a simpler matrix (enriched wafer) produced a strong improvement [29]. In the group supplemented with vegetables, serum β- and α-carotene increased, but no changes were observed in retinol concentrations in serum or breast-milk or in MRDR test results. In our study, we will use several measures of vitamin A status, including serum retinol concentration, the CIC procedure, total dietary vitamin A status, and the deuterated retinol dilution method. It is our hope that the latter procedure will prove to be a reliable index of vitamin A body stores and more sensitive index of vitamin A states in humans.

2.4.3.1. Food source, preparation, and ingestion

In order to minimize non-compliance, mothers will be consulted regarding the fruit/vegetable preferences of their children, and the information will be used in the selection of fruits/vegetables for the intervention phase. The foods will be obtained in bulk from the International Institute of Rural Reconstruction (IIRR), a pioneer of bio-intensive gardening in the Philippines, which will be contracted by the Nutrition Center of the Philippines to grow the vegetables. A vegetable viand containing 1.4 mg of β-carotene will be consumed with rice during lunch at school. Oil will be used in the food preparation. Two 0.5-mg β-carotene portions of fruits/vegetables will be consumed as snacks during the mid-morning and mid-afternoon.

The control group will be given an equivalent amount of calories in the form of cereal/rice products, and jam containing no vitamin A or carotenes.

Aliquots of homogenates of foods for the experimental and control groups will be analyzed for their carotenoid content by HPLC at the HNRCA, Boston.

3. RESULTS

3.1. In the Philippines

We have identified the study sites (experimental and control) which are two villages of similar socioeconomic characteristics: i) the village of Hukay, in the municipality of Silang, province of Cavite, about 50 km south of Manila, and ii) the village of Santa Elena, in the municipality of SantoTomas, province of Batangas, about 70, km from Manila. The two villages are far apart, thus there will be no sharing of information among villagers regarding the study. They are similar in road conditions, economic status of families, and school population. Secondary nutrition data of the Silang Health Center in 1995 show that Hukay has a high prevalence of moderately and severely underweight pre-schoolchildren. Hukay Elementary School records (1995) reveal that 48 (i.e., 27%) of the 179 school children in Grades 1 to 6 are moderately and severely underweight. In Santo Tomas, Batangas, a study done by the Nutrition Center of the Philippines in 1993 showed that ~30% of schoolchildren have serum retinol levels <20 μg/dL [11].
We have submitted the study for approval by the Ethics Review Board of the Philippine Council for Health and Research Development, and approval has been granted.

We have conferred with local government, school, and health authorities in the two villages.

We are arranging for the procurement of fruits and vegetables from the IIRR. The Nutrition Center of the Philippines will contract the IIRR to grow the vegetables for the study, in order to have a single source of vegetables, thus minimizing variability in their nutrient composition.

The Nutrition Center of the Philippines has many years of experience in conducting nutrition surveys and vitamin A studies in children, including clinical and biochemical assessments for xerophthalmia, CIC measurements, and dietary intake assessments [3,6,8,11].

3.2. In Boston

We are making arrangements for the synthesis of tetradeuterated- and octodeuterated retinyl acetate.

The HNRCA laboratory in Boston routinely measures serum retinoids and carotenoids by HPLC, and has done initial studies of GC/MS retinol measurements [25,26].

4. PLANS FOR FUTURE WORK

We hope to complete all preliminary work and the field work in the Philippines during the first 18 months (November 1995 to April 1997), and all analyses (biochemical, dietary, cytological, statistical) during the following year.

The school calendar in the Philippines starts in mid-June and ends in mid-March. In order to avoid disruption of the study during the period of school vacation, we plan to start the study at the beginning of the school year. Screening procedures and blood drawing will be done in July/August 1996. Serum samples (n~300) will be transported to Boston and analyzed for retinol in September/October 1996. Children identified to have low serum retinol levels will be invited to participate in the study. They will be dewormed in November - one month prior to the start of the study. Baseline isotope dilution tests will be conducted in December 1996, and the intervention phase will proceed from January to March 1997. We hope to complete the post-intervention isotope dilution tests and other tests by April 1997. We expect to finish the writing-up of study results for publication in scientific journals by late 1997 or early 1998.

An evaluation of the efficacy of nutrition programmes aimed at improving the health of vulnerable populations is essential. Factors affecting the bioavailability of carotenoids and vitamin A are not well understood. We hope that this study is the first of many more future collaborative studies on carotenoid and vitamin A nutrition between the Nutrition Center of the Philippines and the HNRCA at Tufts University in Boston, Massachusetts, USA.
REFERENCES


TABLE I. PREVALENCE OF SIGNS AND SYMPTOMS OF VITAMIN A DEFICIENCY BY REGION: PHILIPPINES, 1993

<table>
<thead>
<tr>
<th>Region</th>
<th>Nightblindness</th>
<th>Bitot’s spot</th>
<th>Corneal Ulceration</th>
<th>Corneal Scar</th>
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<td>I. Ilocos</td>
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PHILIPPINES 1.1 0.1 - 0.0

*Project Area

Source: National Nutrition Survey, Food and Nutrition Research Institute, 1993
Abstract

HIV infected pregnant women have been shown to be at risk for vitamin A deficiency. Poor vitamin A status has been shown to be a risk factor for transmission of HIV virus from mother to infant. It is however, difficult to assess the vitamin A status in such women by conventional methods such as serum retinol because of the effect of the acute phase response on serum retinol and because of the haemodilution that occurs during pregnancy. This study therefore sets out to determine more accurately the vitamin A stores of HIV infected pregnant women by means of the stable isotope dilution method. In addition the stable isotope dilution method will be used to validate the usefulness of breast-milk retinol status as an indicator of vitamin A status and its correlation with the infant’s vitamin A status.

1. SCIENTIFIC BACKGROUND

1.1. Vitamin A status of women and children

Vitamin A (VA) deficiency has emerged as a major cause of childhood mortality and morbidity in the developing world [1,2]. VA deficiency may also affect adults and may directly or indirectly influence child health by its effect on maternal health and on breast-milk vitamin A levels in breast fed babies.

VA supplementation in children has been reported to have a positive effect on mortality reduction [3]. Supplementation has generally been achieved by programmes aimed at infants. However, it may be prudent to consider improving the vitamin A status of mothers by supplementation as a means of protecting the vitamin A status of breast feeding infants. If successful, the vitamin A status of infants will be protected during a period when infections have their highest toll.

Serum retinol (SR) levels among pregnant women in the developing world are reported to be lower than those among their counterparts in the developed world [4]. In addition, SR levels decrease during pregnancy [5], but increase to non-pregnant levels during the post partum period (within 24-48 hours) [6]. It needs to be noted that serum retinol levels during pregnancy may be misleading due to the haemodilution that accompanies the later stages of pregnancy. However, it may also suggest that ingested vitamin A is used less efficiently in pregnancy, or, that the requirement is higher than previously thought. Previous studies report a decreased mean intake of VA [7] and mean levels of SR in the deficient (<20 ug/dL) or marginal range (20-30 ug/dL) among lactating women in the developing world [8,9]. Thus, the picture of VA status among pregnant women in the
developing world seems to be one of deficiency. The situation among pregnant women locally is unclear.

Newborns have lower SR and carotene levels than their mothers, possibly due to limited transplacental transfer [10,11]. Liver stores are low in the early months of life. Preterm babies have lower SR and liver stores of VA than term babies [10].

The serum retinol level is under homeostatic control over a broad range of body stores, and reflects body stores only when these are very low or very high. Thus, it is not a reliable indicator of VA deficiency in individuals. A further disadvantage for assessing individuals is that SR levels are decreased by acute and chronic underlying infections.

Breast-milk VA (BMA) concentration is a unique indicator of VA status in both the mother and the breast fed infant. The level of vitamin A in breast-milk is associated with its fat content, post natal age and gestational age at birth. Although, expressing BMA content relative to the fat content improves the accuracy, the sensitivity and specificity are not high enough to identify VA deficiency in individuals.

Isotope dilution (ID) is a relatively new technique that has promise as the most sensitive, specific and reliable test outside of liver biopsy, for assessing the VA status of individuals [12]. The principle of this technique is the following; an oral dose of deuterated retinyl acetate is administered and is allowed to mix with the body vitamin A stores. The ratio of labelled to unlabelled serum retinol is used to calculate the pool size by tracer dilution principles. This method of assessment of VA status has not previously been used among pregnant women and their babies in developing countries. The VA status of pregnant women in South Africa needs to be studied using this more sensitive and specific test.

1.2. Vitamin A status of women and children

Transmission of HIV from mother to infant occurs in 10-52% of pregnancies. The higher rates of transmission have generally come from studies in developing countries, and particularly from Africa. Many factors contribute to the risk of transmission, including viral load, maternal immunological and clinical status and whether the baby is breastfed or not.

VA may also have an important role to play in the transmission of HIV from mother to infant. HIV infection is accompanied by multiple nutritional deficiencies [13], including VA [14]. In an unpublished study from an underprivileged population in South Africa, HIV infected women had significantly lower VA levels at delivery when compared to uninfected controls [unpublished observations, Coutsoudis, A]. Semba et al has shown that maternal VA deficiency may contribute to mother to infant transmission of HIV [15]. In this study those women with the lowest levels of VA had the highest rates of MTI transmission of HIV. VA deficiency has also been implicated as a risk factor for progression to AIDS in adults, and increased mortality in adults, during infancy and in the perinatal period. Therefore, VA deficiency among women and infants in developing countries may be an important risk factor for the increased rate of transmission and the more rapid progress to clinical disease among children in developing countries as opposed to Europe and the United States.

Supplementation of VA has been shown to increase serum VA levels in lactating women of low socio-economic status [16] and to increase concentration in breast-milk. It may also increase levels in pregnant women, and thereby diminish the risk of transmission.

MTI transmission of HIV may occur during pregnancy, near or at the time of delivery or during breastfeeding. Current evidence suggests that the majority of transmission occurs
close to or at the time of delivery. Possible sites for transmission during delivery include the placenta and the genital tract (including the vaginal mucosa).

VA supplementation has been shown to improve and maintain epithelial integrity. VA deficiency causes keratinization of epithelia such as the respiratory epithelium and the cornea. Keratinization of the vaginal epithelium cells will be examined by means of immunocytochemistry.

We postulate that the biological effects of VA that diminish the risk for transmission of HIV include stimulation of the immune system and improved mucosal integrity, particularly, the vaginal mucosa. The mechanisms by which VA may have an effect in reducing mother to infant transmission of HIV needs to be elucidated.

1.3. Policy implications of study

The results of this study have wide public health implications. The confirmation by sensitive techniques of VA deficiency in HIV infected pregnant women has implications for their further management. VA supplementation in this group may reduce morbidity and short term mortality. If supplementation of pregnant women is associated with improved VA status in babies, and if it is found to efficiently protect the VA status of breast fed infants, it will have profound public health implications in reducing infant mortality and morbidity. In addition, the optimal tests for population surveys of VA status may be identified. This will allow more accurate identification of target populations for VA supplementation and to assess the efficacy of such interventions.

2. PURPOSE

To assess the vitamin A stores of HIV infected pregnant women and relate this to serum and breast-milk retinol and to the structure of the vaginal epithelium.

3. OBJECTIVES

a. To assess the liver stores of vitamin A, in HIV infected women in the third trimester of pregnancy, by stable isotope dilution methods.

b. To assess the vitamin A status of these women by serum retinol concentration.

c. To determine acute phase protein concentration in these women.

d. To determine the correlations between serum retinol concentration; acute phase protein concentrations and liver stores of vitamin A.

e. To assess immune status and examine the structural and functional integrity of vaginal epithelium in these women.

f. To determine the correlations between serum retinol concentration; liver stores of vitamin A; immune status and vaginal epithelium structural and functional integrity.

g. To determine breast-milk retinol concentrations at 1 month post-delivery.

h. To determine serum retinol concentrations at 1 month post-delivery.
To assess liver stores at 1 month post-delivery by stable isotope dilution method.

To correlate serum retinol with breast-milk retinol and liver stores.

To determine serum retinol in the infant at 1 month post-delivery and correlate with maternal vitamin A status.

To determine hepatic stores of vitamin A in the infant at 3 and 6 months after their mothers have received either a placebo or 200 000 IU of vitamin A (at delivery) in order to determine how long maternal vitamin A supplementation will protect infant vitamin A stores.

4. STUDY DESIGN AND METHODS

4.1. Study population

Pregnant HIV infected women during the third trimester of pregnancy will be recruited at the antenatal clinic of King Edward Hospital, Durban, South Africa. These women will be part of a larger trial designed to test the effect of vitamin A supplementation in reducing mother to infant transmission of HIV. They will be randomly allocated to receive either vitamin A or placebo. Vitamin A treatment will involve a daily oral dose of vitamin A (5000 IU retinyl palmitate and 20 mg b-carotene) from ± 28 weeks gestation until delivery. At delivery they will receive 200 000 IU of vitamin A or placebo.

4.2. Sample Size

A pilot study will be conducted involving 60 women, approximately 30 who will be vitamin A supplemented as part of the main trial and 30 who will be on placebo.

4.3. Baseline measurements before supplementation

At ± 24 weeks gestation, women will have vaginal scrapings collected and will receive a 3 mg dose of tetra-deuterated retinyl acetate (d4-R). Blood samples will then be drawn at 3 intervals within 21 days of receiving the d4-R dose in order to estimate the ratio of d4-R to retinol to assess liver stores. During this period serum retinol concentrations will be determined by High Pressure Liquid Chromatography. In addition acute phase protein levels will be determined and immune status will be assessed. The vitamin A supplementation will commence 21 days after the d4-R dose is given.

4.3.1. Structural & functional integrity of vaginal mucosa

A commercially available endocervical brush shall be used to obtain scrapings from the lateral vaginal walls. Cells obtained shall be suspended in 4% gluteraldehyde with 1% formaldehyde for electron microscopy. This specimen shall be centrifuged and the pellet embedded in resin. Ultrathin slices shall be examined by electron microscopy. Further scrapings from the vagina shall be smeared onto slides for light microscopy and immunocytochemistry using monoclonal antibodies to keratin 14 and 19 as primary antibodies.

Vaginal secretions shall be obtained by aspiration of the posterior fornix of the vagina with a polypropylene pipette. If less than 2 ml of secretions are obtained, 5 ml of saline shall be instilled prior to aspiration. The saline shall contain a fixed concentration of LiCl (100 mEq/l). After aspiration, the mixture shall be homogenized and a subsample (0.5 mls) shall be used to determine the concentration of Li. From this determination, the volume of
secretion aspirated can be determined. Viral load of HIV in the vaginal secretions will be
determined and will be related to viral load of HIV in the blood as a measure of functional
integrity of the vaginal epithelium.

4.3.2. Isotope dilution method

The plasma isotopic ratios of d4-R/retinol will be estimated by GC-mass spec
according to the method of Handelman et al. [17].

4.4. Post-intervention measurements

One week post-delivery the women will receive either 3 mg of d-8 retinyl acetate
(vitamin A treatment group) or 1.5 mg of d-8 retinyl acetate. 21 days later bloods will be
drawn for determination of plasma retinol, and ratios of d8-R/retinol to estimate liver stores
as in the method described above. Breast-milk will be collected for determination of breast-
milk retinol concentrations. The infant's vitamin A status will be assessed by serum retinol
concentration and correlated with maternal plasma retinol and liver stores.

4.4.1. Breast-milk retinol concentrations

The retinol in breast-milk shall be analyzed in a similar fashion to serum retinol. In
addition, the fat content shall be determined by a simple "creamatocrit" method [18]; the
"creamatocrit" is measured and the amount of fat determined by reference to a standard
curve of fat concentrations established by a chemical method.

4.4. Estimation of liver stores in infant at 3 and 6 months of age

At 3 and 6 months of age the liver stores will be estimated by the isotope dilution
method discussed in order to determine the effect of maternal supplementation (200 000
IU at delivery) on hepatic stores of vitamin A in the infant. At 3 months the d-4 retinyl
acetate will be used and at 6 months the d-8 retinyl acetate will be used. The results will
be compared with those in infants whose mothers were not supplemented.

5. ETHICS

Approval for the study has been obtained from the Ethics Committee, Faculty of
Medicine, University of Natal.
REFERENCES

IMPACT OF DIETARY VITAMIN A INTERVENTIONS ON TOTAL BODY STORES IN THAI LACTATING WOMEN

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²Johns Hopkins University, Baltimore, MD, USA

Abstract

Vitamin A deficiency (VAD) is increasingly being recognized as a public health problem among pregnant and lactating women in developing countries. Programs which emphasize increasing the production and consumption of locally available carotenoid-rich foods represent a long-term strategy to prevent VAD and promote reproductive health. Consumption of carotenoid-rich foods has long been considered to maintain adequate vitamin A status; however, this tenet has been recently challenged by studies showing lack of apparent improvement of vitamin A status to dietary interventions that routinely provide foods containing provitamin A carotenoids. This lack of effect could be real or due to poor responsiveness of presently used biochemical indicators of vitamin A status. This proposed study will be a randomized trial to evaluate the efficacy of consuming provitamin A-rich foods in one prepared, on-site meal per weekday for 3 months on total body vitamin A stores and other aspects of vitamin A status in marginally nourished lactating women in rural Northeast Thailand. Approximately 400 lactating women, 2-18 months post-partum, will be screened in the population for marginal vitamin A status by a tier of indicators beginning from low intake or history of night blindness or impaired dark adaptability followed by low serum retinol (<0.70 umol/L). Their marginal vitamin A status will be confirmed by the modified relative dose response test (MRDR > 0.06) at baseline period. Assuming a prevalence of low serum retinol of ~20%, 90 women will be identified and recruited, matched by serum retinol and month post-partum (± 1 month) and randomized in a block fashion into three groups (n = 30) to receive daily cooked (fat-added) meal and snack with (1) dark green leafy and yellow/orange vegetables and fruits, (2) beta-carotene-enriched rice chips and (3) non-enriched rice chips. Groups 1 and 2 will receive ~3.6 mg of beta-carotene (providing ~600 µg RE) per day. Prior to and following the intervention hepatic vitamin A reserves will be estimated by isotopic dilution techniques (over a 4-week equilibration period), and other indicators of vitamin A status (serum and breast-milk retinol and carotenoid panel, MRDR, CIC, dark adaptometry). In addition, serum C-reactive protein and maternal anthropometry will be measured. Food consumption data based on 24-hour recall for 3 randomized days will be collected every 2 weeks to assess routine intakes of vitamin A, fat and other nutrients. Morbidity will be monitored on a weekly basis throughout the study. Between-group comparisons will provide a basis for (1) estimating the adequacy of local diets to improve or maintain total body stores of vitamin A in women during lactation and (2) assessing the validity and responsiveness of widely used measures of vitamin A status in this high-risk group.
1. SCIENTIFIC BACKGROUND AND SCOPE OF THE PROJECT

Vitamin A deficiency affects approximately 230 million children in developing countries [1]. Severe or clinical deficiency results in progressive stages of eye lesions or xerophthalmia leading to blindness [2]. Mounting evidence revealed that mild to moderate or subclinical deficiency posed individuals at increased risk from infectious diseases and childhood mortality [3]. In addition, vitamin A deficiency is increasingly being recognized as a public health problem among pregnant and lactating women in developing countries [4,5].

Vitamin A deficiency has existed in Thailand for many years particularly among rural people and low-income dwellers. While severity of the problem has declined in most regions, several surveys have revealed a high prevalence of subclinical vitamin A deficiency in rural areas of Thailand [6]. A 1990 survey in Northern and Northeastern Thailand, for example, indicated that approximately one-fifth of preschool children in these regions experience subclinical vitamin A deficiency as evidenced by low liver stores estimated by the relative dose response (RDR) and abnormal conjunctival epithelium examined by conjunctival impression cytology (CIC) [7]. A different picture, however, emerged from the lower region of Southern Thailand where cases of infant xerophthalmia and keratomalacia in preschool children were reported in 1991 and 1992 [8]. Since then, a vitamin A supplementation program has been launched and targeted at infants and preschool children in high risk areas. This intervention in coordination with food-based dietary intervention efforts have resulted in no new reported cases of clinical vitamin A deficiency. At present, based on WHO's new prevalence classification, Thailand has been categorized as a country with moderate subclinical vitamin A deficiency [9]. In regard to vitamin A deficiency among pregnant and lactating women, a small scale survey conducted in 1987 found a history of night blindness among 8 and 10% of pregnant and lactating women, respectively [6]. Recent dietary surveys in 1989 and 1991 of lactating women in the northeast Province of Ubon showed the average vitamin A intakes to be 253 and 333 RE/day representing 25 and 33% of Thai RDAs, respectively (Viriyapanich T, unpublished observations). These data suggested that a considerable proportion of lactating women in the rural northeast are at risk of vitamin A deficiency and may benefit from dietary intervention programs.

Increasing production and consumption of locally available carotenoids-rich foods represents an appropriate long-term strategy to prevent chronic, subclinical vitamin A deficiency and promote reproductive and child health. Rural communities depend primarily on plant sources rich in provitamin A carotenoids (dark green leafy vegetables and yellow/orange fruits and vegetables) for their vitamin A supply. Although consumption of carotenoid-rich foods has long been considered to maintain adequate vitamin A status, this tenet has been recently challenged by a study showing lack of apparent improvement of vitamin A status to dietary interventions that routinely provide provitamin A carotenoids in the form of dark green leafy vegetables [10]. These findings could be due to poor responsiveness of presently used biochemical indicators of vitamin A status or the closer-to-normal vitamin A status (and thus, non-responsiveness) of the studied population. To clarify the extent of impact on vitamin A status to be expected from improved dietary provitamin A intake, we propose to conduct a randomized trial to evaluate the efficacy of consuming provitamin A-rich foods as on-site meals on total body vitamin A stores and other aspects of vitamin A status in marginally nourished lactating women in rural Northeast Thailand.

2. SPECIFIC AIMS

The goals of this study are to increase total body stores of vitamin A, vitamin A status and breast-milk vitamin A levels of marginally nourished lactating women through regular consumption of a local diet rich in provitamin A carotenoids; to demonstrate the
utility of estimating total body vitamin A stores by stable isotope dilution; and to
demonstrate the ability of stable isotope methods to provide a "gold standard" for
evaluating other measures of vitamin A status in high-risk populations.

Specifically, this randomized trial will:

a. Assess the efficacy of daily meals containing provitamin A-rich vegetables and fruits
   given five days per week for twelve weeks in improving total body vitamin A stores
   and other aspects of vitamin A status in lactating women;

b. Test the ability of stable isotope dilution to show changes in apparent total body
   vitamin A stores and breast-milk concentrations of vitamin A in response to known,
   varied dietary intakes of beta-carotene and;

c. Evaluate the sensitivity and specificity of other common measures of vitamin A
   status (i.e., serum retinol, dark adaptometry, impression cytology, breast-milk retinol)
   against estimates of total body stores, and changes in body stores, of vitamin A
   obtained by deuterated retinol measurement in lactating women.

3. METHODS

This randomized, controlled dietary vitamin A trial will be conducted in several rural
villages in the remote northeastern provinces of Ubon and Srisaket where anemia and low
vitamin A intakes during pregnancy and low infant birth weight are widely prevalent.

3.1. Subjects

All lactating women, 2-18 months post-partum, as well as pregnant women during
the third trimester, living in 50 to 80 villages (n≈400 women) will be screened for risk of
vitamin A deficiency by being positive for any of the following: reporting a dietary vitamin
A intake of < 50% of the Thai RDA, providing a current history of night blindness, or
testing positive for impaired dark adaptation using a portable instrument developed at
Institute of Nutrition at Mahidol University, Thailand (INMU). Approximately 180 high-risk
women are expected to be identified during this screening. This group will undergo further
testing for marginal vitamin A status as evidenced by serum retinol concentrations below
0.70 umol/L. Inadequate vitamin A nutriture will be confirmed later by estimating apparent
vitamin A stores by the modified relative dose response (MRDR) (11). It is expected that
slightly more than half, or approximately 100 women, will have an MRDR value ≥ 0.06
(indicative of marginal liver stores) and will be eligible for study. The purpose, procedures,
expected benefits and risks will be explained to these women following guidelines approved
by Thai and US institutional review boards and they will be asked to participate in the trial.
Based on prior research experience in this region we anticipate that ~90% of all eligible
women (n = ~90), will provide signed consent and agree to be enrolled into the trial. On
enrollment, these women will receive a demographic (e.g., age, reproductive history) and
socio-economic status (e.g., education, literacy, household assets, etc.) assessment.

3.2. Intervention trial

The trial will evaluate the impact of different dietary regimens on maternal stores of
vitamin A and other aspects of vitamin A status. Total body vitamin A stores will be
assessed in enrolled women by deuterated retinol (d4) isotopic dilution and dietary intake
will be estimated during a 42-day dilution period. Thereafter, subjects will undergo baseline
assessment for vitamin A, hematologic and anthropometric status, morbidity, and other
exposures. They will then be randomized to receive one of three dietary groups [daily meal
and snack with (a) provitamin A-rich vegetables and fruit providing ~3.6 mg beta-carotene, (b) beta-carotene enriched rice chips (~3.6 mg beta-carotene), or (c) non-enriched rice chips as a control regimen) five days per week for 12 weeks. Following the intervention period, vitamin A, hematologic and anthropometric status will be reassessed and a repeat 42-day isotope dilution measurement will be carried out to determine the absolute and relative impacts of each dietary regimen on total body vitamin A stores and vitamin A status.

3.4. Baseline assessment

Prior to beginning the intervention all enrolled women will be given a single tracer dose of 5 mg of deuterated retinol (d4) in an oil base in two gelatinous capsules. On the same day subjects will be evaluated for dietary intake (focusing on food sources of preformed and pro-vitamin A carotenoids), anthropometry and recent morbidity. Blood will be drawn 3, 28 and 42 days later, centrifuged and the serum frozen on the same day for later determination of isotope dilution. Twenty-four hour dietary intake on randomly selected 3-day intervals will be periodically assessed during this time in order to assess relative constancy in vitamin A intake (and thus, assumed total body vitamin A pool) among groups during this time period. Once isotope levels are obtained subjects will be rank-ordered according to their fractional disappearance [11] of tracer on day 3 of the post-dosing "window" during which plasma disappearance curve is sensitive to stores. Liver stores will be estimated from the Furr equation [13] based on obtained levels of the d4 tracer in serum on day 28 and the fractional catabolic rate derived from the terminal slope calculated for the interval between days 28 and 42. On day 42 subjects will also undergo baseline assessment for vitamin A status (serum and creatocrit-adjusted breast-milk retinol and carotenoids), serum C-reactive protein level, hematologic (Hb and serum ferritin) and anthropometric status (weight, height, mid-upper arm circumference and tricipital and subscapular skinfolds thicknesses) and dark adaptometry by light intensity detection at INMU and pupillary response threshold detection at Johns Hopkins University (JHU) [14]. They will then be randomized to treatment groups prior to beginning the dietary trial 1-3 days later.

3.5. Randomization

Following baseline assessment (day 42), subjects will be matched by serum retinol concentrations and month postpartum (+/- 1 month) and then randomly assigned to one of three dietary treatment groups, blocked on every 15th assignment. There will be n, = 30 per group, a number that will be sufficient to test differences in estimated total body stores and other outcome measures among groups using large-sample approximation tests of statistical significance. Randomization is expected to provide, on average, experimental groups that are comparable with respect to estimated total body vitamin A stores, vitamin A, hematologic status and anthropometric status as well as potential confounding influences.

3.6. Dietary Intervention

Subjects will be given a daily meal and a take-home snack every weekday for 12 weeks according to one of three dietary regimens:

1) A rice or noodle meal with cooked dark green leafy vegetables (e.g., ivy gourd, Chinese swamp cabbage, amaranth) or other provitamin A-rich vegetables (e.g., carrot, yellow sweet potato, ripe pumpkin) plus a carotenoid-rich fruit snack (e.g., ripe papaya, ripe mango) providing a total of ~3.6 mg of beta-carotene per day;
2) A rice or noodle meal containing non-provitamin A vegetables with beta-carotene enriched rice chips plus a take-home snack with no provitamin A (providing a total of ~3.6 mg of beta-carotene per day); or

3) A rice or noodle meal containing non-provitamin A vegetables with non-enriched rice chips plus a take-home snack with no provitamin A.

All three dietary menus will contain approximately 8-12 grams of added fat per day. Aliquots of each group of carotenoid-rich foods will be analyzed for carotenoid profile (i.e., beta-carotene, alpha-carotene, lutein/zeaxanthin, cryptoxanthin and lycopene).

During this 12-week intervention period dietary assessment by semi-monthly, random, 3-day 24-hour recalls will be conducted to monitor and adjust outcomes for consumption patterns, including levels of dietary substitution that may be practiced by subjects. In addition, weekly histories of morbidity, medicinal use, smoking and alcohol use will be recorded to adjust for possible influences on dietary intake and vitamin A stores and status.

3.7. Follow-up assessment

At the end of the intervention subjects will be reassessed for serum and breast-milk retinol and carotenoid levels, serum acute phase protein level (C-reactive protein), Hb and serum ferritin, MRDR, dark adaptation (by both the INMU and JHU methods) and anthropometry. A subsequent tracer dose of 5 mg of deuterated retinol (d8, in order to avoid confounding of values by residual d4 concentrations from baseline) will be administered to all subjects, followed by blood sampling at 3, 28 and 42 days following the loading tracer dose. As done at baseline, subjects will again be evaluated for dietary intake (to account for potential vitamin A pool dilution) and recent morbidity during this follow-up period.

3.8. Laboratory analyses

Serum and breast-milk retinol and carotenoids and food carotenoid profile will be determined by high performance liquid chromatography, serum C-reactive protein and ferritin by ELISA technique and hemoglobin by standard chemistry method. All analyses will be carried out at the INMU laboratory facilities in Salaya Campus following standard and quality control procedures using both internal and certified reference materials to monitor protocol and control a day-to-day variation.

Isotope specimens will be placed under liquid nitrogen and shipped to the Center for Human Nutrition at the Johns Hopkins School of Public Health for analysis by gas chromatographic-mass spectrometry (VG TRIO 3 GC-MS).
REFERENCES


PART IV:

Agenda of Research Coordination Meeting

List of Participants
AGENDA

Thursday 14 Dec. 1995

09.30-10.00 Welcome
Adoption of Agenda
Administrative arrangements

10.00-10.30 Overview of purpose and scope of CRP
10.30-11.00 Introductions and brief summary of research to be undertaken within the CRP
11.00-11.15 Break
11.15-12.30 Continuation of introductions
12.30-12.45 Welcome by USDA/WHNRC
12.45-14.15 Lunch break
14.15-14.45 Overview of WHO Multi-Centre Vitamin A Supplement Programme
(B. Underwood)
14.15-15.00 Discussions
15.00-16.30 Measurement of vitamin A stores using tracer dilution methods
(M. Haskell)
16.30-17.00 Discussions
18.00 Group dinner at local restaurant

Friday 15 Dec. 1995

09.00-10.30 Moderator: J. Lembcke
Project presentations:
Evaluating interventions to improve vitamin A status in children
Peru-EPI M. Haskell, J. Lembcke
Philippines F. Solon
Israel R. Reifen
10.30-10.45 Break
10.45-12.30 Continuation of project presentations
Moderator: A. Coutsoudis
Peru - school breakfast programme J. Lembcke
India P. Sudhakaran
China Shian Yin
12.30-14.00 Lunch break
14.00-15.00 Continuation of project presentations
Moderator: A. Coutsoudis
15.00-17.00 Physiological data of relevance to interpreting vitamin A in children
(B. Underwood)

Discussion:
Identifying standard protocol matters for vitamin A status assessments in children
Saturday 16 Dec. 1995

09.00-10.30 Isotope tracer dilution theory
Assumptions and R & D needed for using the method to measure vitamin A stores in pregnant women and lactating women (M. Green)

10.30-10.45 Break

10.45-12.15 Moderator J. Ribaya-Mercado
Project presentations:
Measuring vitamin A stores in pregnant or lactating women
South Africa A. Coutsoudis
Thailand E. Wasantwisut
India P. Sudhakaran

12.15-14.00 Lunch break

14.00-15.30 Continuation

15.30-16.40 Discussions

Sunday 17 Dec. 1995

09.30-10.30 Measuring vitamin A status in the field in pregnant or lactating women: Biological issues (K. West)

10.30-11.00 Discussions

11.00-11.15 Break

11.15-12.30 Intrinsic labeling of pro-vitamin A carotenoids for human studies of pro-vitamin A carotenoid bioavailability (P. Nair)

12.30-13.00 Discussions

13.00-14.30 Lunch break

Monday 18 Dec. 1995

09.00-10.30 Finalize protocol revisions: Child-oriented studies

10.30-11.00 Break

11.00-12.30 Final protocol revisions: Maternal-oriented studies
Delegation of responsibility for writing final report of the meeting

12.30-14.00 Lunch break

14.00-16.40 USDA Laboratory tour
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