SEASONAL VARIATION IN SERUM 25-HYDROXYVITAMIN D IN HEALTHY FAIRBANKS, ALASKA RESIDENTS: RELATION TO DIET AND SUNLIGHT EXPOSURE

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SEASONAL VARIATION IN SERUM 25-HYDROXYVITAMIN D
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RELATION TO DIET AND SUNLIGHT EXPOSURE

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By
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Chapter 1

Introduction

1.1 Historical background

Disturbances of calcium metabolism in humans due to seasonal changes in photoperiod have been noted in the literature for many hundreds of years (Norman 1979). The skeletal disease associated with low exposure to solar radiation, rickets, was first described by Daniel Whistler of Leidon in the Netherlands in 1645, and independently by Professor Francis Glisson of London in 1650 (Hess 1929). But Hess has suggested that the early writings of Soranus, a physician in the Greco-Roman era (circa 100-200 A.D.) gave the first recognizable account of this debilitating bone disease.

Little progress was made on understanding the etiology of the disease rickets until the early 20th century, but it did become clear that the disease was not limited to western Europe, and Hess wrote in 1929 that the incidence of rickets was equivalent to a map of sunlight deficiency. More recently though, many papers have
emerged describing rickets and osteomalacia in countries not thought of for deficiencies in sunlight, such as Greece, Saudi Arabia, India, South Africa and the southern United States (Lapatsanis et al. 1968, Sedrani et al. 1983, Raghuramulu and Reddy 1982, Pettifor et al. 1978, Omdahl et al. 1982).

Norman (1979) has outlined the history of proposed causes of the disease rickets, which ranged from genetic and infectious factors to lack of exposure to UV rays of sunshine. It wasn't until the early 20th century that deficiencies of certain essential dietary factors, micronutrients, were proposed as the cause of certain disease states. In 1913, McCollum and Davis published their classic study which showed that a substance found naturally in butterfat and cod liver oil, which they called vitamin A, was essential to the growth and health of animals. Sir Edward Mellanby was next able to experimentally produce rickets in dogs and then cure the disease with cod liver oil, attributing the recovery to the dietary factor, vitamin A (DeLuca 1979). But McCollum recognized that vitamin A was not the only growth factor found in cod liver oil. He performed an experiment whereby he destroyed the vitamin A growth factor by heating the oil in the presence of oxygen, but the resultant oil retained its anti-rachitic properties
McCollum coined the term for this fat soluble organic micronutrient as vitamin D.

The confusing connection between sunlight and the fat soluble vitamin D found in cod liver oil was made in studies by Chick et al. in 1923, who found they could cure rickets in humans with either sunshine or with exposure to artificial UV light. This anomaly was finally explained when Goldblatt and Soames in 1923 showed that UV irradiation of rachitic rats produced a substance in their livers which could be extracted and fed to other rachitic rats to correct their disease (DeLuca 1979).

The first chemically pure vitamin D compounds were isolated in 1932 by Windaus et al. and independently by Askew et al., from an irradiated plant steroid called ergosterol (DeLuca 1979). Fortification of milk with ergosterol, otherwise known as vitamin D₂, was begun soon after in the United States and this led to a dramatic reduction in the incidence of rickets in this country. Subsequently during the 30's and 40's, another anti-rachitic compound was isolated from fish oils and later from irradiated human skin. This compound came to be known as vitamin D₃ and its chemical name is cholecalciferol. This compound is now used more often to fortify foods but both vitamins D₂ and D₃ have been found
to be equally potent in humans. Experiments in this area are still ongoing.

While experiments into the chemical production, isolation and purification of vitamin D continued, a better understanding of the physiological importance of the vitamin was taking place. In the 1930's and 1940's the role of vitamin D in the intestinal absorption of calcium and phosphorus, and its importance in promoting renal tubule reabsorption of calcium, was being discovered. Vitamin D was thought to play a direct role in normal bone formation, but further study determined that it aided bone formation indirectly by elevating serum calcium and phosphorus. During the 1950's, with the production of radiolabeled vitamin D compounds, it was discovered that vitamin D also assisted, along with parathyroid hormone, in the mobilization of calcium and phosphorus from bone (DeLuca 1979, Norman 1979).

1.2 Vitamin D: photoproduction, regulation, metabolism and function

1.2.1 Photoproduction of vitamin D₃ in skin

The production of vitamin D₃ from pro-vitamin D (a cholesterol precursor) in the epidermis and dermis has long been thought as the major way in which humans get
their vitamin D (Stanbury 1981). It has recently been shown that the skin is also a site for regulation of endogenous vitamin D production (Holick 1981, Holick et al. 1981). Exposure of skin to UV-B radiation (spectral range 290-315 nm) produces previtamin D₃ from the provitamin D, 7-dehydrocholesterol (Fig. 1). In order for this to happen, a photon of light is absorbed by 7-dehydrocholesterol which causes the cleavage of the C₉-C₁₀ bond of this molecule. With the addition of body heat, previtamin D₃ isomerizes to vitamin D₃.

Excess sunlight exposure does not lead to excessive and potentially toxic amounts of this vitamin in circulation, but rather leads to the production of two chemically inert products, lumisterol and tachysterol (Holick et al. 1981). If levels of previtamin D₃ drop, both lumisterol and tachysterol can absorb light and again become previtamin D₃ thus helping to maintain a steady stream of vitamin D production. Excess lumisterol and tachysterol can be sloughed off during the natural skin turnover processes (Holick 1985a). Thus there are adaptive mechanisms in the skin to regulate a somewhat steady flow of newly formed vitamin D into circulation. The story on production of vitamin D compounds in the skin is not yet complete and Holick's group recently identified a new provitamin D₃, 24-dehydroprovitamin D₃ in rat and
Fig. 1. Vitamin D endocrine system, from A.W. Norman 1985.
human skin (Holick 1985b). The role this compound plays in vitamin D metabolism has not yet been discerned.

A recent study has shown that in aging there is a greater than two-fold reduction in the skin's capacity to produce previtamin D$_3$. MacLaughlin and Holick (1985) demonstrated that this reduced capacity is due to a more than two-fold reduction of the precursor molecule, 7-dehydrocholesterol in the epidermis but not in the dermis of elderly subjects. This decline could not be explained due to the decrease in total epidermal mass found in the elderly.

1.2.2 Metabolism and regulation of vitamin D

Since the early 1970's it was known that vitamin D was not the biologically active form of the vitamin but that it underwent two successive hydroxylations, one in the liver and one in the kidney before it became a more polar, active metabolite. Vitamin D produced in the skin, or absorbed in the intestine, moves into circulation bound with high affinity to a well identified D-binding protein (a globulin protein).

Radiotracer studies have shown that vitamin D is quickly transformed in the liver to 25-hydroxycholecalciferol (25-OHD$_3$) or 25-hydroxyergocalciferol (25-OHD$_2$), collectively represented here as 25-OHD, by a liver
microsomal vitamin D-25-hydroxylase. The activity of this enzyme is somewhat enhanced in D-deficient animals but its regulation seems to be weak, and is not yet fully understood (DeLuca 1979). The largest circulating metabolite of vitamin D is 25-OHD and this metabolite is thought to represent most closely the body stores of vitamin D (Adams et al. 1982, Mawer 1980). 25-OHD is not considered to be biologically active except in non-physiologically high serum levels, such as those found in vitamin D intoxication through over consumption of vitamin D supplements.

Once 25-OHD has been formed, it moves on the same D-binding protein to body storage sites, found in adipose tissue, skeletal muscle and bone (Mawer et al. 1972). In addition, some 25-OHD undergoes a highly regulated second enzymatic hydroxylation in the kidney to become what is now thought of as a vitamin D hormone, 1,25-(OH)$_2$vitamin D. When extracellular Ca++ is lowered, parathyroid hormone is released into circulation, and this in turn stimulates the activity of the mitochondrial enzyme, 25-hydroxyvitamin D-1-hydroxylase (DeLuca 1979). Once serum Ca++ levels have been elevated by the actions of the newly formed D metabolite, PTH levels drop and the 25-hydroxyvitamin D-1-hydroxylase activity falls (Fig.1). This mitochondrial hydroxylase is also inhibited by its
product, \(1,25-(OH)_2D\) (Henry and Norman 1984). Elderly osteoporotic patients were recently shown to have a markedly reduced response to infused PTH and were thus thought to have a reduced ability to adapt to diets varying in calcium content (Slovik et al. 1981)

Other hormones found to stimulate production of the active vitamin D hormone are: growth hormone, prolactin, insulin, estrogens, and androgens. The final known effector of the 1-hydroxylase enzyme is serum inorganic phosphate (\(P_i\)) levels. If serum \(P_i\) levels fall, the 1-hydroxylase enzyme is stimulated independent of parathyroid hormone. The mechanism of this stimulation is not yet understood but is thought to be mediated through growth hormone (Gray and Lemann 1985).

1.2.3 Function of vitamin D hormone

Production of \(1,25-(OH)_2D\) whether from lowered serum calcium, or phosphorus levels, or by stimulation from the previously mentioned hormones leads ultimately to increased serum calcium and phosphorus levels due primarily to its action in the intestine in stimulating the absorption of calcium and phosphorus. Thus \(1,25-(OH)_2D\) comes into play in hypocalcemic or hypophosphatemic conditions, whereas another hormone comes into play in hypercalcemic or hyperphosphatemic conditions.
conditions, namely calcitonin from the thyroid gland. Hypercalcemia is much less common than hypocalcemia and as such, calcitonin is not thought to be of major importance in the maintenance of calcium homeostasis in adults.

Because vitamin D is metabolized to its active form in one part of the body (the kidney mitochondria), and is then transported in blood to its site of action in another part of the body, it is known as a hormone. In addition, like other steroid hormones, there are receptors for the active vitamin D hormone which are usually located in the cytoplasm and nuclear membranes of target cells and it is thought that this hormone functions by triggering the production of messenger RNA to produce specific proteins which change the activities of target cells. Anthony Norman has coined the term for the active vitamin D metabolite as the calcium homeostatic steroid hormone (Norman 1979).

It has long been known that vitamin D improves intestinal calcium absorption (DeLuca 1979), and it is now known that this occurs by at least two mechanisms. The active vitamin D hormone has been shown to stimulate the production of vitamin D-dependent calcium-binding protein (CaBP) in the brush border membrane cells, as well as to change the permeability of the brush border membrane to ionized calcium by stimulating changes in the phospholipid
composition of these membranes. These two actions of the vitamin are thought to assist in a major way in the transfer of calcium from the gut into circulation (Wasserman et al. 1984). The way in which vitamin D hormone assists in the transport of $P_i$ into circulation is not yet fully understood but probably also involves the production of a transport protein.

Receptors for vitamin D hormone have also been found in bone, and the hormone has been found to stimulate the production of still another CaBP, as well as an alkaline phosphatase in bone. This may be part of the way in which it assists in the mobilization of calcium and phosphorus from bone, but there are other mechanisms, some of which involve parathyroid hormone, which have not yet been elucidated. A third way in which vitamin D hormone has been found to assist in the elevation of serum calcium is by the stimulation of proximal renal tubule reabsorption of calcium (Henry and Norman 1984). Consequently, receptors for the hormone have also been located in the kidney.

One current avenue of vitamin D research is focusing on the identification of new receptor sites in the body for vitamin D hormones. Additional receptors for vitamin D hormone have been isolated in many different tissues including the parathyroid, pancreas, pituitary, and
parotid glands, mammary tissue, and placenta (Fig.1). The function of vitamin D hormone in these many and varied tissues is not fully understood, but the next ten years of research in this area will probably lead to discoveries that vitamin D hormone plays a much wider role in maintenance of body calcium homeostasis than was ever first imagined.

1.3 Vitamin D and calcium status in Alaskans

Vitamin D is unique among the vitamins in that the primary source of this vitamin comes from its photoproduction in the skin which is quite limited in winter in subarctic and arctic Alaska. When light limits photoproduction, dietary sources of vitamin D then become important and vitamin D takes its place among the vitamins as a requirement in the diet for normal growth and health. Earlier human nutrition surveys in Alaska have not included biochemical analyses for serum vitamin D metabolites nor the analysis of diet habits to assess the average vitamin D intake (Heller and Scott 1967, Sauberlich et al. 1972, Bell and Heller 1978). Most of the comprehensive surveys occurred in the late 1950's and 1960's before the major vitamin D metabolites had been isolated or could be easily analyzed, and before food composition handbooks included the vitamin D contents of
at least some vitamin D-containing foods. Chronically low intakes of the related nutrient, calcium, have been reported for some Alaskan groups over the years (Draper 1977, Draper 1980, Knapp and Panruk 1978, Stetson 1985) and recent surveys have shown that low calcium intakes are a prevalent problem in Fairbanks (Milan et al. 1982, Stetson 1986).

There is however a need to assess the vitamin D status of Alaskans as short day lengths and extreme cold ambient temperatures of the long arctic and subarctic winters must limit endogenous vitamin D production in this region. A recent survey comparing annual UV radiation values in Fairbanks to lower latitude locations in the United States, showed that Fairbanks received 65% or less than the annual UV levels reported for the lower latitude cities (Table 1). In addition, because both Alaskan and Canadian Eskimos have been found to have an earlier onset and greater rate of aging bone loss when compared to Caucasians from Wisconsin (Mazess and Mather 1974, 1975), the vitamin D status of these groups and other groups should be studied to discover if the presumably seasonally lowered vitamin D status coupled with usually low calcium intakes, could be contributing to the accelerated rates of aging bone loss seen in these Alaskans (DeLuca 1979).
Table 1. Mean daily values of total UV radiation for summer, winter and the year for eight sites in the USA (kJ m\(^{-2}\) day\(^{-1}\)).

<table>
<thead>
<tr>
<th>Site</th>
<th>APRIL-SEPT SUMMER</th>
<th>OCT-MARCH WINTER</th>
<th>ANNUAL VALUE</th>
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<tbody>
<tr>
<td>FAIRBANKS, ALASKA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1980</td>
<td>619</td>
<td>120</td>
<td>369</td>
</tr>
<tr>
<td>1981</td>
<td>626</td>
<td>113</td>
<td>370</td>
</tr>
<tr>
<td>CORYALLIS, OREGON</td>
<td>974</td>
<td>350</td>
<td>662</td>
</tr>
<tr>
<td>DAVIS, CALIFORNIA</td>
<td>926</td>
<td>424</td>
<td>675</td>
</tr>
<tr>
<td>HONOLULU, HAWAII</td>
<td>1117</td>
<td>775</td>
<td>946</td>
</tr>
<tr>
<td>ANN ARBOR, MICHIGAN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1980</td>
<td>788</td>
<td>368</td>
<td>578</td>
</tr>
<tr>
<td>1981</td>
<td>788</td>
<td>368</td>
<td>578</td>
</tr>
<tr>
<td>1982</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAN ANTONIO, TEXAS</td>
<td>994</td>
<td>589</td>
<td>792</td>
</tr>
<tr>
<td>ATLANTA, GEORGIA</td>
<td>988</td>
<td>572</td>
<td>780</td>
</tr>
<tr>
<td>ALBANY, NEW YORK</td>
<td>816</td>
<td>358</td>
<td>587</td>
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To date no epidemiological studies have been conducted to assess the incidence of rickets or osteomalacia (adult rickets) in Alaska but there is some anecdotal evidence of vitamin D deficiency, and bowed legs, presumably caused by vitamin D and/or a calcium deficiency, are uncommon but noticeably present in both native and non-native lifelong residents of Alaska. Recently Mazess et al. (1985) published the first paper on the vitamin D status of an Alaskan group. Their study assessed the major circulating vitamin D metabolites at the end of winter (in March) in a group of older adult Aleuts living on St. Paul Island, and found 25-OHD levels to be within a normal, non-rachitic range when compared to other United States studies. Another recent study of Inuits and Caucasians living in Arctic Canada found the Caucasians and Inuit children with a greater incidence of lowered vitamin D levels than the Inuit adults (von Westarp et al. 1981). These differences were attributed to vitamin intake differences between the groups.

1.4 Major goals and hypotheses of this study

The major goal of this study was to assess the vitamin D status of a healthy group of Fairbanks residents over a one year period, to provide baseline data for future comparisons to other Alaskan groups at greater risk
for vitamin D deficiency, i.e. the elderly, darker-skinned individuals, indoor workers, pregnant and lactating females, infants and children, and alcoholics. A second objective was to ascertain whether diet or sunlight (UV) exposure play a more important role in affecting circulating 25-OHD over the changing seasons of the year. The final objective of this study was to give recommendations as to what are sufficient amounts of sunlight exposure and vitamin D intake to maintain adequate serum levels of this nutrient, out of the deficient range. The major hypotheses tested in this study were:

1. Fairbanks residents should exhibit an annual seasonal fluctuation in serum 25-OHD, the largest pool of vitamin D metabolites, with minimum levels in winter and spring months due to lower UV radiation levels leading to less photoproduction during these months.

2. Those individuals with higher yearly mean sunlight exposure should have higher yearly mean serum 25-OHD levels than those with lower yearly mean sunlight exposure.

3. Those individuals consuming a diet high in vitamin D should have significantly higher yearly mean serum 25-OHD levels than those consuming a diet low in vitamin D.
4. Low UV radiation levels during the long Fairbanks winter will lead to the reduction of serum 25-OHD into rachitic levels, at least during some of the winter months, in individuals consuming diets low in vitamin D.

5. A seasonal reduction of serum calcium levels in spring due to lowered vitamin D status will lead to an eventual elevation of vitamin D hormone (1,25-(OH)₂D) production in response to PTH secretion and consequently to seasonal changes in serum calcium and phosphorus.
Chapter 2

Materials and methods

2.1 Human subjects

Forty-seven healthy adult volunteers were chosen from respondents to a poster announcement for the study (Fig. 2) and from respondents who heard the same message read on a local educational radio station (KUAC). Subjects were initially screened over the telephone, and finally interviewed in person by myself before being accepted into the study. With the use of the Volunteer Questionnaire (Appendix A) potential volunteers were interviewed and excluded from the study if they had any history of liver, kidney, intestinal, or dietary diseases. Volunteers were presumed to be eating their usual diet but dietary supplements containing vitamin D and/or calcium were not to be consumed over the course of the year long study.

Those who wanted to use sunlamps or attend tanning centers were excluded from the study. Yearlong residency in Fairbanks was a requirement for participation, but
Interested people are needed to participate in a one year study to assess the effects of long dark winters on the vitamin D and calcium levels of Fairbanks residents. Each month volunteers will be asked to fill out 3-day food intake and outdoor activity records, and donate blood samples for a one year period beginning in September 1983.

For more information contact: Meredith Tallas
Room 313 Irving Bldg
University of Alaska
Ph. 474-7164 or 479-3593

Please call as soon as possible but not later than Sept. 10, 1983.

_institute of Arctic Biology, University of Alaska, Fairbanks_

Fig. 2. Poster announcement for Fairbanks vitamin D study.
those taking short term vacations out of state (or out of country) were not excluded.

All forty-seven participants were Caucasian as the respondents were overwhelmingly from this ethnic group. The volunteer group was mainly composed of students, staff and faculty of the University of Alaska, Fairbanks (65° N Lat). The average age of the group, which included nineteen males and twenty-eight females was 34 years (range: 22-66). Only two participants were more than fifty years old, and both were males.

2.2 Consent forms and study approval

This study was approved by the Human Experimentation Committee of the University of Alaska and all participants signed Volunteer Consent Forms indicating their informed consent (Appendix B, C).

2.3 Blood sample collection

An experienced medical technician, Ms. Bonnie Bond, was hired to take the overnight fasting blood samples (12 cc by vacutainer) over the course of the study. The samples were taken between 08:00 and 12:00 hr in the first two weeks of each sampling month, starting October 1983 through September 1984. Participants were offered juice and a snack after the blood samples were withdrawn.
Volunteers were sent monthly reminder cards of upcoming sampling dates approximately one week before the actual sampling date.

2.4 Serum preparation

The whole blood was allowed to clot for 25 to 60 minutes and after centrifugation the serum was pipetted into four equal-sized portions, stored frozen (-20° C) in covered Omni-Vials (Wheaton Scientific), and secured with taped name labels to reduce potential water loss. Samples were kept frozen until analyzed the following year and each volunteer's serum set was processed together to minimize interassay variation.

2.5 Volunteer record-keeping

Each volunteer was given a notebook which included blank Sunlight Exposure record forms (Appendix D) and blank Food and Beverage Diary forms (Appendix E) which they filled out for four consecutive days (three weekdays and one weekend day) each month. Record-keeping dates were assigned; however, on occasion, volunteers altered these dates, typically if they forgot to start recording on a set date, became ill or were out of town unexpectedly. Participants were discouraged from keeping records while on vacation, or if ill. I made an attempt
to alternate between assigned sequences so that a Sunday was included in one month and a Saturday in the next.

Sunlight exposure forms were filled in by the volunteers to record their exposure to sunlight, from sunrise to sunset. Exposure to sunlight while behind auto or window glass wasn't noted as this light does not contain the required wavelengths for photoproduction of vitamin D. Food and Beverage Diary forms were filled in to record the types and quantities of all foods and beverages (except water) consumed in each 4-day period.

Volunteers were encouraged to fill out the forms daily rather than completing them at the end of the 4-day time period from memory. I interviewed each participant monthly to verify accuracy and completeness of both the sunlight exposure and food records when they came to give blood samples and records. I made an attempt not to influence either food choice or outdoor activity behaviors. Sometimes in-person interviews weren't possible and the individual was later interviewed over the phone.

Each participant was offered his or her own set of measuring cups to keep at home to use in estimating volumes of food consumed. Cardboard color food models were used during the interviews to prompt volunteers to make accurate estimates of food quantities consumed.
Compliance with record keeping was fairly good in the first nine months of the study, but participation became less regular as the study continued.

2.6 25-(OH)vitamin D separation and analysis

2.6.1 Separation of vitamin D metabolites

Both 25-OHD$_2$ and 25-OHD$_3$ were separated from all other vitamin D metabolites in a procedure developed by Reinhardt et al. (1984). I visited Dr. Bruce Hollis, one of the co-authors of the above paper, at Case Western Reserve University in Cleveland, Ohio in November 1984 to learn the separation procedure under Dr. Hollis' guidance.

On the day of extraction, thawed serum samples (0.125 ml) were added to sterile saline (0.875 ml) to equal a final volume of 1.0 ml (Fig. 3). Five hundred cpm of 25-hydroxy[26(27)-methyl-$^3$H]cholecalciferol (Amersham, TRK.396, specific activity= 22 Curies/mmol) in 20 μl ethanol were added to each serum, saline blank and plasma control sample, as well as to a counting vial containing 10 ml scintillation cocktail (4g PPO +50 mg POPOP/L toluene), to monitor recovery. The samples were vortexed lightly and allowed to stand for 10 minutes.

The solid phase extraction of serum protein was next done by adding one volume (1.0 ml) of acetonitrile to each
Fig. 3. Separation scheme for vitamin D metabolites.
serum sample. The tubes were vortexed vigorously for 20 seconds and centrifuged for 10 min at 1500x g to remove protein precipitate. After centrifugation, the supernate was pipetted into a tube containing 0.5 ml 0.4 M K₂HPO₄, pH 10.6, and vortexed vigorously for at least 30 sec. Tubes were spun at 1500x g for 10 min and the extract was applied with a pasteur pipette to a pre-washed C-18 Sep-Pak (Water's Assoc., Inc., Milford, MA).

Both new and used C-18 Sep-Paks were prepared before use by rinsing with the sequential addition of 3 ml isopropanol, 3 ml hexane, 3 ml isopropanol, 3 ml acetonitrile, and 5 ml distilled H₂O. Silica Sep-Paks were rinsed with 5 ml methanol, 3 ml isopropanol, and 6 ml hexane. This conditioning procedure allowed repeat use of Sep-Pak cartridges at least five times without significant loss in metabolite recoveries. A Sep-Pak cartridge rack (Water's Assoc.) was used to hold eight Sep-Paks so that eight samples could be simultaneously processed. A vacuum pump attached to the cartridge rack was used to pull all solvents through the Sep-Pak columns. All organic solvents used were HPLC grade.

Excess salt was removed from samples by washing the cartridges with 5 ml distilled water. Polar lipids were removed by washing the cartridges with 3 ml of methanol-water (70:30). Vitamin D metabolites were then eluted
with 3 ml acetonitrile and this fraction was dried under a stream of nitrogen at 40°C in a waterbath.

The vitamin D extract was resuspended in 0.5 ml hexane-isopropanol (97:3) and applied to a silica Sep-Pak cartridge (Water's Assoc.). The cartridge was rinsed with 7 ml of hexane-isopropanol (99:1) and the 25-OHD fraction was eluted with 8 ml of hexane-isopropanol (97:3). This fraction was dried, as above, under nitrogen.

Immediately after drying the extract was resuspended in 200 μl absolute ethanol and vortexed lightly. Extracts were sealed tightly with rubber stoppers and stored at -20°C for up to one week before analysis. On the day of analysis 200 μl of the extract was added to a scintillation vial containing 10 ml of a previously described scintillation cocktail and the samples were counted at least 5 min to calculate 25-OHD recovery.

2.6.2 Analysis of total 25-OHD

The analysis of 25-(OH)vitamins D₂ + D₃ is typically accomplished with a sensitive protein-binding assay. The method is based on the preliminary saturation of a specific D-binding protein, usually a globulin protein found in rat serum, with [³H]25-(OH)cholecalciferol. Upon subsequent addition of unknown serum extracts containing 25-OHD, some labeled compound is displaced into solution
during an incubation. The displaced (unbound) fraction is removed with dextran-coated charcoal, the samples centrifuged and the charcoal-free supernate assayed by liquid scintillation counting. Counts from unknown serum samples are then compared to counts from known 25-OHD standards.

The initial method used for analysis of 25-OHD was a modification of a competitive protein-binding assay developed by Preece et al., 1974. I observed this method in 1981 in Dr. Mike Holick's Vitamin D Lab, Massachusetts General Hospital, Boston. The method reproduced in Alaska proved to have a large interassay variation and some assays gave anomalously high estimates of 25-OHD. Therefore a second and very similar assay was chosen which I initially observed and ran in Dr. Bruce Hollis' lab at Case Western Reserve University in 1984. This assay was a modification of Hollis' earlier assay and differs from the Preece assay in type of buffer and bovine serum albumin used, incubation time and use of swine gelatin with the rat serum D-binding protein (Hollis, 1984). This method gave me an intra-assay coefficient of variation= 8.0% (n=8) and an interassay coefficient of variation= 13.5% (n=40). The method is described below.

Weanling Holtzman rats were grown on a Purina #5825 rachitogenic rat chow and deprived of UV radiation for
eight weeks after weaning when they were killed by blows to the head and the blood was removed from the hearts. Serum was extracted and diluted 1:100 in borate buffer (pH 8.4) and stored in 2 ml aliquots in omni-vials at -20° C. This batch of diluted serum was used as the vitamin D-binding protein (DBP) source over the next two year period. Rat serum DBP, a globulin protein, was found to be stable when stored this way for at least two years (Preece et al. 1974).

25-hydroxy [26(27)-methyl-3H] cholecalciferol (spec. act. approx. 22 Curies/mmmole) supplied in toluene/ethanol (9:1 v/v) was purchased from Amersham. The toluene/ethanol solvent was evaporated with nitrogen and absolute ethanol was used to quantitatively dilute the tracer to 500 and 5000 cpm in 20 μl ethanol. Diluted tracer was covered with argon and stored in airtight glass Reacti-vials (Pierce Chemical Co.) at -20° C for use for up to four months.

Purified crystalline unlabelled 25-hydroxycholecalciferol was obtained as a generous gift from Dr. Milan Uskokovic of Hoffmann-LaRoche Company. A stock 25-OHD solution was prepared by dissolving a very small amount of the dessicated crystals in 2 ml of 100% ethanol. A scanning spectrophotometer was used to test each concentrated preparation for purity before dilution. The
test scan was run between 190 and 300 nm to look for a peak (264 nm)/trough (228 nm) absorbance ratio of 2.0. Solutions which showed deviations from this ratio after subtraction of ethanol blanks were considered impure and discarded (personal communication, Vitamin D Lab, Mass. General Hospital).

Dilutions of the stock 25-OHD were made with chilled ethanol to produce 10 μg/ml and 64 ng/ml using the peak absorbance at 264 nm, the molar absorptivity (∊₂₆₄=18,300 liters mole-cm⁻¹), the formula weight (402 g/mole) and Lambert-Beer's Law (A=∊bC, Flaschka et al., 1969). Dilute solutions were stored in airtight glass containers, covered with a layer of argon, and kept at -20° C for up to four months until used.

Donated plasma from the Fairbanks Memorial Hospital blood bank was collected in August 1983, pooled and stored in sealed Omni-vials in 2 ml portions and frozen at -20° C to be used as plasma control sample over the next two years. The 25-OHD metabolites are considered to be very stable when stored at -20° C (personal comm., Dr. Bruce Hollis).

A stock buffer solution for assay procedures was prepared in advance by dissolving 6.19 g of boric acid, 2.03 g of NaOH, adding 2.3 ml of conc. HCl and bringing up to 1 L with distilled H₂O to give a final concentration of
0.10 M borate buffer (pH 8.4). This solution was stored at 5° C until completely used.

On the day of analysis several reagent solutions were prepared fresh. The dextran-coated charcoal separation solution was made by adding 1.0 g Norit A activated charcoal (Sigma Co., C5260) with 0.1 g Dextran T-40 (Pharmacia Fine Chem.) and 30 mg of globulin-free bovine serum albumin (Sigma, A7638) to 100 ml distilled water and stirring slowly in the cold (5° C) for at least two hours (overnight also proved acceptable). This solution was then poured into 30 ml plastic centrifuge tubes and centrifuged at 800x g for 10 minutes. The cloudy supernate was decanted and the pellets resuspended in 100 ml stock borate buffer with 30 mg of the BSA again added.

Unlabelled 25-OHD standard solutions were made by serially diluting 0.4 ml stock 25-OHD (64 ng/ml) 1:2 repeatedly seven times in chilled absolute ethanol to produce a standard curve which ranged from 3200 pg/ml to 25 pg/ml. The standard solutions were kept on ice until used.

Borate buffer used to dilute rat serum (just thawed) was initially treated by heating (but not boiling) 30 mg of swine gelatin (Sigma Co.) in 100 ml of stock buffer and then chilling it before use. Rat serum was diluted to
1:18,000 in the above prepared borate buffer on the day of use. This solution was kept on ice.

The following components were added to iced borosilicate tubes (10x75 mm) according to the schematic found in figure 4:

1. Tracer addition:
   Approx. 5000 cpm $[^{3}\text{H}]25\text{-OHD}$ in 20 µl ethanol were added to the bottom of every assay tube.

2. Standard or Sample addition:
   2.1. 50 µl of the above prepared standards was added to each labeled standard tube.
   2.2. 50 µl of above Sep-Pak extracted unknown serum samples was added to each duplicate unknown tube.
   2.3. 50 µl of Sep-Pak extracted plasma control was added to each replicate "PC" tube.
   2.4. 50 µl absolute ethanol was added to each triplicate "Total" and "Zero" tube.
   2.5. 50 µl concentrated 25-OHD (10 µg/ml) was added to each triplicate non-specific binding tube ("NSB" tubes).

3. Buffer and rat serum D-binding protein addition:
   3.1. 0.5 ml of borate buffer with swine gelatin and rat serum was added to all tubes except "Total" tubes.
**Fig. 4.** Tube additions for 25-(OH)vitamin D protein-binding assay.

<table>
<thead>
<tr>
<th></th>
<th>2000 cpm</th>
<th>STANDARD</th>
<th>CONCENTRATED</th>
<th>UNKNOWN</th>
<th>BORATE BUFFER</th>
<th>BORATE BUFFER/CHARCOAL</th>
<th>DEXTRAN CHARCOAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[3H]25-ohD_3</td>
<td>25-ohD_3</td>
<td>ethanol</td>
<td>25-ohD_3</td>
<td>serum</td>
<td>binding protein</td>
<td>pH 8.4 suspension</td>
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<td>UNKNOWNS</td>
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<td></td>
<td>20 UL</td>
<td>50 UL</td>
<td>50 UL</td>
<td>50 UL</td>
<td>50 UL</td>
<td>0.5 ML</td>
<td>0.5 ML</td>
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<td>X</td>
<td>X</td>
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<td>X</td>
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<td>X</td>
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<td>STANDARDS</td>
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<td>X</td>
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<tr>
<td>TOTALS</td>
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<td>X</td>
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<tr>
<td>ZER0</td>
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<td>X</td>
<td>X</td>
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<tr>
<td>NON-SPECIFIC BINDING</td>
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<td>X</td>
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<td></td>
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<td>X</td>
</tr>
</tbody>
</table>
3.2. 0.5 ml of buffer alone (with no rat serum) was added to each Total tube.

4. Tubes were lightly vortexed, covered with parafilm and incubated at 5° C for at least two hours. The incubation can go overnight and would result in decreased non-specific binding.

5. Charcoal Addition: 0.2 ml of prepared dextran-coated charcoal suspension was added to all incubation tubes except Total tubes. The tubes were vortexed and kept at 5° C for 20 min. After 10 min the tube racks were shaken lightly and placed at 5° C for the additional 10 min.

6. All tubes were centrifuged at 3000x g for 15 min and supernates decanted into 20 ml glass scintillation vials containing 4.5 ml of Instagel scintillation fluid (Packard). Both % recovery and assay samples were counted sequentially for at least 5 min using a Beckman LS 7500 Liquid Scintillation counter. All counts were recorded on a Data General S140 Eclipse computer which was interfaced with the LS 7500 counter.

2.6.3 Calculations

Counts from both the % recovery and assay samples were corrected for quenching by counting a separate quench
set for both assay and % recovery samples. A basic program was written for the Eclipse computer to utilize the output data from the LS 7500 to transform cpm to dpm, produce a standard curve in the form of a logit/log plot, and calculate the final unknown values corrected for both non-specific binding, serum dilution, blank reading and losses incurred during extraction.

Competitive protein-binding assays (CPB) and similar radioimmunoassays (RIA) commonly utilize a logit/log transformation of the standard data (Chard 1982). In many applications this transformation results in a linear inverse standard curve (Fig.5). The basic program written to make this logit/log transformation was modeled after an RIA program from the Hewlett-Packard HP-41C calculator Clinical Lab and Nuclear Medicine Program Pack (Hewlett-Packard, Palo Alto, Ca). A logit/log standard curve has the proportion of tracer bound expressed as a percentage of the zero standard on the y axis and the log of the standard concentration on the x axis.

\[
y = \text{logit} \left[ \frac{(B_i-\text{NSB})}{(B_o-B_i)} \right] = \ln \left[ \frac{B_i-\text{NSB}}{B_o-B_i} \right]
\]

and \( x = \log C_i \)

where

\[
\ln = \text{natural logarithm} \text{ (base 2.17828)}
\]

\[
\log = \text{common logarithm} \text{ (base 10)}
\]

\( B_i \) = average of replicate counts for the ith standard
Fig. 5. Logit/log standard curve for 25-(OH) vitamin D assay.
\[(i=1,2\ldots n)\]

NSB = average of replicate counts for non-specific binding

Bo = average of replicate counts for zero concentration

Ci = concentration of the ith standard \((i=1,2,\ldots n)\)

The concentration of the unknown is then calculated as:

\[Cu = 10^x\]

where

\[Cu = \text{concentration of unknown serum}\]

\[x = \frac{1}{m} \left[\ln (Bu-NSB/Bo-Bu) - b\right]\]

\[m = \text{slope}\]

\[b = \text{y intercept}\]

Bu = average of replicate counts for an unknown

2.7 Analysis of other blood metabolites

A subsample of paired serum samples for March (end of winter) and September (end of summer) from twenty-five year-round residents who did not take south island vacations were, in addition to 25-OHD, examined for several metabolites which are commonly examined when assessing vitamin D status.
2.7.1 Analysis of 1,25-(OH)₂D

During the fall 1984 visit to Cleveland, I took the 25 pairs of serum samples and quantitatively separated the total 1,25-(OH)₂D using the previously described Sep-Pak separation procedure. The 1,25-(OH)₂D fraction was collected by passing 9 ml of hexane:isopropanol (85:15) through the same silica Sep-Pak which had previously been rinsed to remove the 24,25-(OH)₂D and the 25-OHD fractions.

The 1,25-(OH)₂D fraction was dried under nitrogen at 40° C, resuspended in 100% ethanol and analyzed by Dr. Hollis using a recently developed nonequilibrium radioreceptor assay (Reinhardt et al. 1984). The 1,25-(OH)₂D receptor was isolated from fresh calf thymus. Four pairs of samples were lost during processing giving results for 21 pairs in pg/ml.

2.7.2 Analysis of ionized calcium

A second subsample of the above 25 serum pairs was also examined in Hollis' lab for ionized calcium using a Radiometer ICA 1 Ionized Calcium Analyzer and Radiometer pre-mixed solutions. This automated analyzer corrected final values for pH to give mmol Ca++/liter serum at pH 7.4.
2.7.3 Analysis of total calcium

At the Fairbanks Memorial Hospital in Fairbanks, Alaska, I assisted Dr. Bill Vorkink in analysis of another frozen subsample of the same 25 serum pairs for total calcium, albumin, total inorganic phosphorus and alkaline phosphatase activity. In all hospital and laboratory analyses, each pair of samples was analyzed together to avoid interassay variation for the pair.

Total calcium was measured colorimetrically at 570 nm using an Abbott VP autoanalyzer and Abbott reagents. The assay was based upon serum calcium reacting with 0-cresolphthalein complexon in an alkaline solution to produce a measurable purple chromophore. Calcium values obtained by this method are thought to be comparable with those obtained by atomic absorption spectrophotometry and results are expressed as mg/100 ml. One pair of samples was lost during processing.

2.7.4 Analysis of serum albumin

Serum albumin was also measured colorimetrically on an Abbott VP autoanalyzer using Abbott A-gent reagents. The test was based on the ability of albumin to bind to bromcresol green dye, and the absorbance of the dye-
protein complex was measured at 630 nm. The final values were expressed as g albumin/100 ml serum.

2.7.5 Analysis of total inorganic phosphorus

Inorganic phosphorus was analyzed on the above serum pairs by measuring the absorbance at 340 nm of a phosphomolybdic acid complex. The analysis was done using a Technicon kit (# T01-1303) and samples were measured with a Technicon RA-1000 autoanalyzer. Values were expressed as mg inorganic phosphorus/100 ml.

2.7.6 Analysis of alkaline phosphatase activity

Total serum alkaline phosphatase activity (AP) was measured by a simple colorimetric test based upon the enzyme's ability to hydrolyze a monoester of orthophosphate. The substrate utilized was p-nitrophenyl phosphate and the absorbance at 415 nm was measured to quantify the rate of production of p-nitrophenolate. An A-Gent alkaline phosphatase test kit from Abbott Laboratories was used and measurements were made on an Abbott VP autoanalyzer. Final values are expressed as International Units/liter serum, where one International Unit of AP activity is defined as the amount of activity which will catalyze the conversion of one mmole of substrate/minute.
2.8 Measurement of total UV radiation

Total ultraviolet (UV) radiation was measured using an Eppley UV photometer which detects UV light between 290-385 nm. This measurement was wider than the band necessary for the production of pre-vitamin D in the skin (MacLaughlin et al. 1982) but it gave a relative measure of UV radiation throughout the year. Continuous daily measurements in watt-hours m⁻² were made for the first nine months of the study, from the top of the Elvey building at the Geophysical Institute of the University of Alaska, Fairbanks campus (Dr. Gerd Wendler's lab). But data collection was stopped in July 1984 due to the loss of funding for their project. Therefore UV data from the previous four years was pooled and used to complete the yearlong monthly estimates of total UV radiation.

2.9 Analysis of sunlight exposure records

Sunlight exposure diaries were examined to calculate the average daily sunlight exposure in minutes/day for each of the 12 months on all 47 volunteers. The daily time periods examined each month were chosen by taking the time period from one-half hour after sunrise to one-half hour before sunset (to the closest half hour), as determined from the first day of each month (Table 2).
<table>
<thead>
<tr>
<th>Month</th>
<th>Time Sunrise*</th>
<th>Time Sunset</th>
<th>Time Period Sunlight Exposure Tabulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>January 1</td>
<td>10:55 am</td>
<td>2:56 pm</td>
<td>11:30 am to 2:30 pm</td>
</tr>
<tr>
<td>February 1</td>
<td>9:38 am</td>
<td>4:35 pm</td>
<td>10:00 am to 4:00 pm</td>
</tr>
<tr>
<td>March 1</td>
<td>8:01 am</td>
<td>6:08 pm</td>
<td>8:30 am to 5:30 pm</td>
</tr>
<tr>
<td>April 1</td>
<td>6:09 am</td>
<td>7:44 pm</td>
<td>6:30 am to 7:00 pm</td>
</tr>
<tr>
<td>May 1</td>
<td>4:19 am</td>
<td>9:20 pm</td>
<td>5:00 am to 9:00 pm</td>
</tr>
<tr>
<td>June 1</td>
<td>2:34 am</td>
<td>11:07 pm</td>
<td>3:00 am to 10:30 pm</td>
</tr>
<tr>
<td>July 1</td>
<td>2:11 am</td>
<td>11:37 pm</td>
<td>2:30 am to 11:00 pm</td>
</tr>
<tr>
<td>August 1</td>
<td>3:50 am</td>
<td>10:02 pm</td>
<td>4:30 am to 9:30 pm</td>
</tr>
<tr>
<td>September 1</td>
<td>5:31 am</td>
<td>8:10 pm</td>
<td>6:00 am to 7:30 pm</td>
</tr>
<tr>
<td>October 1</td>
<td>7:00 am</td>
<td>6:20 pm</td>
<td>7:30 am to 6:00 pm</td>
</tr>
<tr>
<td>November 1</td>
<td>8:39 am</td>
<td>4:31 pm</td>
<td>9:00 am to 4:00 pm</td>
</tr>
<tr>
<td>December 1</td>
<td>10:19 am</td>
<td>3:02 pm</td>
<td>11:00 am to 2:30 pm</td>
</tr>
</tbody>
</table>

* Data collated by the Fairbanks National Weather Service.
2.10 Analysis of food and beverage diaries

Four day diet diaries were examined for their vitamin D containing foods for the months of October 1983, January, April and July 1984. Several food composition tables were used to compile a comprehensive list of vitamin D contents of foods (Leveille et al. 1983; Pennington and Church 1985; Paul and Southgate 1978). Each individual's quarterly vitamin D intake was computed by comparison to the list and values were expressed as mean intake of International Units (IU)/day ± 1 standard error of the mean, where one IU= 0.025 μg of vitamin D. Computed values were compared to the Recommended Dietary Allowance for adults, or 200 IU/d (National Research Council, 1980).

2.11 Statistical procedures

Repeated measures multivariate analysis of variance (MANOVA) was used to test for monthly variations of serum metabolites, vitamin D intake and sunlight exposure in the forty-seven volunteers (SPSS Inc. 1983). Various between-subjects groupings were examined including sex, occurrence of a south island vacation, level of yearly sunlight exposure, and level of yearly vitamin D intake. Models
which yielded significant seasonal F scores (p < .05) were further tested with the Student-Newman-Keuls (SNK) comparison of monthly means (SAS Insitute Inc. 1985; Steel and Torrie 1980). Significant group effects and interactions were further tested each month by two-way analysis of variance (SAS Institute Inc. 1985) or by SNK comparison of monthly group means. A large sample sign test was used to examine sex differences in sunlight exposure (Freund 1984). Linear regression analysis was carried out on selected combinations of serum metabolite pairs to determine if predictive relationships existed (Plotrax II, Omicron 1983). Statistical significance was indicated in all tests if p values were less than 0.05. Month and group values for all data sets are expressed as mean ± 1· standard error of the mean.
3.1 Seasonal variation in 25-OHD

A highly significant seasonal variation in total 25-hydroxyvitamin D (25-OHD) was found by MANOVA analysis in this group of healthy Fairbanks residents ($p < .001$). Figure 6 shows the lowest monthly 25-OHD value found in March ($23.1 \pm 1.3$ ng/ml) three months after the lowest monthly sum of total UV radiation, and the highest 25-OHD value seen in July ($34.4 \pm 1.5$ ng/ml) one month after the UV peak. Student-Newman-Keuls (SNK) comparisons of monthly means indicated no significant differences in mean 25-OHD levels between October and May but in June there was a significant rise over the March mean ($p < .05$, Table 3). July through September 25-OHD values were not significantly different from each other despite the fall in monthly sums of total UV, but the July mean was significantly higher than the June mean.

The effect of sex and occurrence of south island vacations (to Hawaii or the Bahamas) on monthly 25-OHD
Fig. 6. Seasonal variation in 25-OHD for the full volunteer group.
Table 3. Results of Student-Newman-Keuls (SNK) comparison of ranked mean monthly serum 25-OHD.

<table>
<thead>
<tr>
<th>Month</th>
<th>Mean (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>March</td>
<td>23.1</td>
</tr>
<tr>
<td>February</td>
<td>23.2</td>
</tr>
<tr>
<td>April</td>
<td>23.9</td>
</tr>
<tr>
<td>January</td>
<td>24.2</td>
</tr>
<tr>
<td>May</td>
<td>24.2</td>
</tr>
<tr>
<td>December</td>
<td>24.5</td>
</tr>
<tr>
<td>November</td>
<td>26.3</td>
</tr>
<tr>
<td>October</td>
<td>27.2</td>
</tr>
<tr>
<td>June</td>
<td>29.1</td>
</tr>
<tr>
<td>September</td>
<td>30.9</td>
</tr>
<tr>
<td>August</td>
<td>33.4</td>
</tr>
<tr>
<td>July</td>
<td>34.4</td>
</tr>
</tbody>
</table>

Means underlined by the same line are not significantly different (a = .05)
levels were tested using MANOVA. The analysis showed a significant effect of sex (p < .015) with the yearly mean 25-OHD level for males (29.4 ± 1.4 ng/ml, range: 20.9-39.9) 16% higher than for females (25.4 ± 1.3 ng/ml, range: 13.2-42.6). During the yearlong study the female monthly 25-OHD means were always lower than the male monthly 25-OHD means (Fig. 7), but of the four individuals whose winter 25-OHD values dropped below 10 ng/ml some time during the winter, two were males and two were females (Fig. 8). The lowest 25-OHD value, 7.2 ng/ml, was found in a female in March. Those individuals with yearly mean values less than 20 ng/ml (n=7, mean = 17.0 ± 2.4 ng/ml) were all females and their individual yearly curves are shown in Figures 9 and 10.

MANOVA analysis showed no overall significant differences in 25-OHD between those who took a vacation to southern latitude islands (Hawaii or the Bahamas) during the study year and those who did not, however a significant interaction was found between trip and month (p < .034). Figure 11 illustrates how monthly 25-OHD levels varied in the spring between the two groups and ANOVA found the months of March and April as significantly higher in the island visitor group (March, p < .04, April, p < .009). Eight of the ten island visitors took their trips between December and April during the study year.
Fig. 7. Comparison of male and female mean seasonal variation in 25-OHD. A significant sex effect (p< .015) was found yearlong using MANOVA.
Fig. 8. Seasonal variation in 25-OHD in "at risk" individuals.
Fig. 9. Seasonal variation in 25-OHD for four females with yearly means less than 20 ng/ml.
Fig. 10. Seasonal variation in 25-OHD for three females with yearly means less than 20 ng/ml.
Fig. 11. Seasonal variation in 25-OHD in island visitors versus all other volunteers. The two groups were found to be significantly different in March (p< .04) and April (p< .009).
The yearly mean 25-OHD levels in those who took an island trip versus those who didn't were, respectively, 28.2 ± 2.8 ng/ml (n=10) and 26.7 ± 1.0 ng/ml (n=37). Examples of the variety of individual responses in serum 25-OHD to presumed increase in sun exposure during island visits are shown in Figure 12. Nine out of ten island visitors showed their highest monthly 25-OHD value in the first or second month after return to Alaska, irrespective of the month in which they took their trip.

3.2 Seasonal variation in sunlight exposure

Highly significant seasonal variation in sunlight exposure was found using repeated measures MANOVA (p < .001). Mean monthly values ranged from a minimum of 14.1 ± 2.2 min/d in December to a maximum of 138.6 ± 15.9 min/d in June with a yearly group mean of 64.1 ± 5.7 min/d (Fig. 13). SNK comparisons of monthly means showed that average sun exposure was not significantly different between October and April but that in May and June there were consecutive significant rises in sun exposure over winter values (Table 4). There were no significant differences between the months of June through September with the exception of August which was significantly lower than June and July, but not September (α = .05).
Fig. 12. Examples of monthly variations in 25-OHD in six island visitors.
Exposure To Sunlight per Day

Minutes of Sunlight Exposure/Day

\( \bar{x} \pm S.E. \) \ (n=35-47)

Fig. 13. Mean monthly variation in sunlight exposure (min/d) for the full group.
Table 4. Results of Student-Newman-Keuls (SNK) comparisons of ranked mean monthly sunlight exposure.

<table>
<thead>
<tr>
<th>Month</th>
<th>December</th>
<th>January</th>
<th>November</th>
<th>February</th>
<th>October</th>
<th>March</th>
<th>April</th>
<th>May</th>
<th>August</th>
<th>September</th>
<th>July</th>
<th>June</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (min/d)</td>
<td>14.1</td>
<td>19.5</td>
<td>20.8</td>
<td>21.1</td>
<td>31.9</td>
<td>43.1</td>
<td>51.3</td>
<td>64.6</td>
<td>100.0</td>
<td>128.6</td>
<td>138.0</td>
<td>138.6</td>
</tr>
</tbody>
</table>

Means underlined by the same line are not significantly different (α=.05)
MANOVA test results showed no significant effect of sex, or sex and month interactions on mean monthly sunlight exposure, but a followup large sample sign test indicated there was a significant trend for male monthly means to be larger than female means in ten out of twelve study months (p < .015). Figure 14 shows the seasonal variation in sunlight exposure for males and females with the yearly mean exposure for males, $68.9 \pm 8.8$ min/d (range: 17-157) approximately 13% greater than that for females, $61.0 \pm 7.7$ min/d (range: 14-208).

3.3 Quarterly analysis of diet records

Compilation of monthly four-day diet records revealed that the yearround average daily vitamin D intake was $204 \pm 16$ International Units(IU)/day (n=47, Table 5). A MANOVA test was fit with sex as the between-subjects grouping and results showed no significant seasonal variation in vitamin D intake in either sex (p < .23, Fig. 15). There was however a significant yearlong sex effect (p < .038) with a male yearly mean intake of $246 \pm 30$ IU/day (range: 92-655) being 40% higher than the female yearly mean intake of $175 \pm 14$ IU/day (range: 79-357).

When vitamin D intake was computed on a kg body weight basis, then MANOVA showed no significant sex effect (p < .256) even though the male yearly mean was $3.51 \pm 0.29$
Fig. 14. Comparison of male and female mean monthly variation in sunlight exposure (min/d). Results of a large sample sign test indicated a significant ($p < .015$) trend for male monthly means to be larger than female means in ten out of twelve study months.
Table 5. Daily vitamin D intake (IU/d), results of quarterly diet record analyses.

<table>
<thead>
<tr>
<th>Group</th>
<th>October</th>
<th>January</th>
<th>April</th>
<th>July</th>
<th>Yearly X</th>
</tr>
</thead>
<tbody>
<tr>
<td>All (n=47)</td>
<td>201 ± 22</td>
<td>182 ± 17</td>
<td>216 ± 23</td>
<td>217 ± 20</td>
<td>204 ± 15</td>
</tr>
<tr>
<td>Males (n=19)</td>
<td>259 ± 47</td>
<td>206 ± 24</td>
<td>254 ± 46</td>
<td>264 ± 33</td>
<td>246 ± 30</td>
</tr>
<tr>
<td>Females (n=28)</td>
<td>162 ± 17</td>
<td>165 ± 23</td>
<td>190 ± 22</td>
<td>184 ± 25</td>
<td>175 ± 14</td>
</tr>
</tbody>
</table>

Values are daily means ± s.e.; MANOVA results indicate significant differences between sexes (p < 0.038) but no significant differences between months (p < .23).
Fig. 15. Comparison of male and female quarterly vitamin D intake (IU/d). Results of MANOVA analysis indicated a significant yearlong effect of sex (p< .038) on vitamin D intake with a male yearly mean intake 40% greater than the female yearly mean intake.
IU/kg·d, 19% larger than the female yearly mean of 2.95 ± 0.14 IU/kg·d (Fig. 16). Again, there was no significant seasonal variation in vitamin D intake (p < .083).

When vitamin D intake was finally calculated on a metabolic body weight basis, IU/kg^{0.75}·d (Fig. 17), MANOVA results again showed no significant effect of sex (p < .129). The male yearly mean was 10.37 ± 1.81 IU/kg^{0.75}·d, 26% greater than the female yearly mean, 8.23 ± 1.0 IU/kg^{0.75}·d. This time, however, the MANOVA test gave a marginally significant month effect (p < .055). No significant sex and month interactions with 25-OHD levels were found in any of the three vitamin D intake analyses.

There were no significant differences between quarters or between males and females in their average intake of vitamin D from fortified foods (Table 6). Females received on average, 49.0% of their total D intake from fortified foods and males received 45.6% from fortified foods (e.g. fortified milk, margarines and breakfast cereals).

3.4 Effect of vitamin D intake and sun exposure on serum 25-OHD

Additional MANOVA analyses examined the effects of both amount of vitamin D intake and level of sunlight exposure on monthly serum 25-OHD levels. A MANOVA model
Fig. 16. Comparison of male and female quarterly vitamin D intake (IU/kg·d). No significant differences were found between the sexes in any quarter when results were calculated as IU vitamin D/kg·d.
Fig. 17. Comparison of male and female quarterly vitamin D intake (IU/kg$^{0.75}$.d).
No significant effect of sex was found but a marginally significant (p<0.055) month effect was found using MANOVA analysis.
Table 6. Percentage of IU/d from fortified foods (e.g. vitamin D fortified milk, margarines, and breakfast cereals).

<table>
<thead>
<tr>
<th>Group</th>
<th>October</th>
<th>January</th>
<th>April</th>
<th>July</th>
<th>Yearly X</th>
</tr>
</thead>
<tbody>
<tr>
<td>All (n=47)</td>
<td>43.4 ± 5.0</td>
<td>47.4 ± 4.5</td>
<td>49.6 ± 4.9</td>
<td>50.2 ± 4.3</td>
<td>47.3 ± 3.7</td>
</tr>
<tr>
<td>Males (n=19)</td>
<td>35.4 ± 8.6</td>
<td>44.6 ± 7.6</td>
<td>49.7 ± 7.4</td>
<td>52.6 ± 7.2</td>
<td>45.6 ± 5.9</td>
</tr>
<tr>
<td>Females (n=28)</td>
<td>48.8 ± 5.9</td>
<td>49.3 ± 5.6</td>
<td>49.5 ± 6.5</td>
<td>48.6 ± 5.5</td>
<td>49.0 ± 4.8</td>
</tr>
</tbody>
</table>

Values are means ± s.e.; no significant differences were found between sexes or between months.
was initially fit with D intake as the only between-sample factor. The volunteers were placed in a low D intake group if their yearly mean intake was less than 200 IU/d, the recommended dietary allowance (RDA) for vitamin D. Those who consumed greater than or equal to 200 IU/d were placed in a high D intake group. Test results showed a highly significant month effect (p < .001) and a yearlong difference in 25-OHD between the two D intake groups (p < .001, Fig. 18), but no group by month interactions were found. The yearly mean 25-OHD level in the low D group was 24.2 ± 1.3 ng/ml (n=26, 7 males and 19 females, mean D intake= 134 IU/day) and in the high D group was 30.6 ± 1.2 ng/ml (n=21, 12 males and 9 females, mean D intake= 290 IU/day).

Each D intake group was then split into three sun exposure groups: those who had a yearly mean exposure less than 30 min/d (n=9), those who had from 30 to 90 min/d (n=29) and those who had greater than 90 min/d (n=9). A MANOVA model was fit with both D intake and sun exposure as between-sample factors. A significant D intake effect was again found (p < .009) as well as a significant month effect (p < .001), and a sun group by month interaction (p < .004), but no other group effects or interactions were found. Figure 19 illustrates the monthly 25-OHD levels in all three sun exposure groups and SNK mean comparisons.
Fig. 18. Seasonal variation in serum 25-OHD in low and high vitamin D intake groups. A highly significant ($p<.001$) difference was found yearlong between the two groups using MANOVA analysis.
Fig. 19. Seasonal variation in serum 25-OHD in low, medium and high sun exposure groups. In any given month adjacent group means with a difference greater than 3.1 ng/ml and non-adjacent means with a difference greater than 3.75 ng/ml are significantly different using SNK comparisons of means (α=0.05).
found significant differences between some of the sun
groups only during the spring and summer months. Table 7
gives the yearly mean values of 25-OHD, vitamin D intake,
sun exposure and male/female makeup of the six D
intake/sun exposure groups used in the MANOVA.

3.5 Seasonal variation in related serum metabolites

Table 8 shows the results of MANOVA for some indices
of serum calcium and phosphorus metabolism measured in
March (end of winter samples) and September (end of summer
samples) in a subset of twenty-five non-island visitors.
Both serum ionized calcium and alkaline phosphatase
activity (AP) in paired serum samples were significantly
higher in September than March (p < .001) and of these two
only AP showed a significant sex effect with males having
higher values than females in both months (p < .002). No
significant seasonal changes in serum 1,25-(OH)_2D, total
calcium, serum albumin or total inorganic phosphorus were
found between the paired samples. There was however a
significant sex effect found in the phosphorus values with
females having higher values than males in both March and
September (p < .039). No significant sex and month
interactions were seen for any of these MANOVA analyses.
Table 7. MANOVA analyses group yearly means: 25-OHD, D intake, sun exposure

<table>
<thead>
<tr>
<th>Group</th>
<th>25-OHD (ng/ml)</th>
<th>D intake (IU/day)</th>
<th>sun exposure (min/d)</th>
<th>sample size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low sun</td>
<td>21.4 ± 1.8</td>
<td>132 ± 16</td>
<td>22.3 ± 1.4</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>(2 males/4 females)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low D intake Medium sun</td>
<td>25.5 ± 2.3</td>
<td>131 ± 12</td>
<td>61.4 ± 5.2</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>(3 males/10 females)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High sun</td>
<td>24.1 ± 1.5</td>
<td>142 ± 12</td>
<td>126.9 ± 16.1</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>(2 males/5 females)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low sun</td>
<td>28.7 ± 2.1</td>
<td>256 ± 24</td>
<td>21.9 ± 3.8</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(1 males/2 females)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High D intake Medium sun</td>
<td>30.8 ± 1.5</td>
<td>299 ± 26</td>
<td>56.5 ± 4.3</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>(9 males/7 females)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High sun</td>
<td>30.9 ± 4.1</td>
<td>267 ± 43</td>
<td>111.0 ± 19.1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>(2 males/0 females)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± standard error
Table 8. Results of other serum metabolite analyses
March and September sample pairs (n=21 to n=25 pairs)

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Mean ± S.E.</th>
<th>Range</th>
<th>MANOVA Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,25-(OH)₂ Vitamin D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pg/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>x total (n=21) = 29.28 ± 1.66</td>
<td>27.82 ± 1.87</td>
<td>March: 14.10-51.13</td>
<td>sex n.s.</td>
</tr>
<tr>
<td>x females (n=12) = 28.56 ± 1.97</td>
<td>29.62 ± 2.91</td>
<td>September: 14.53-53.56</td>
<td>month n.s.</td>
</tr>
<tr>
<td>x males (n=9) = 30.23 ± 2.97</td>
<td>25.42 ± 1.90</td>
<td>sex x month n.s.</td>
<td></td>
</tr>
<tr>
<td>Total Calcium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg/dl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>x total (n=24) = 9.18 ± 0.08</td>
<td>9.07 ± 0.09</td>
<td>March: 8.59-9.95</td>
<td>sex n.s.</td>
</tr>
<tr>
<td>x females (n=13) = 9.14 ± 0.12</td>
<td>9.00 ± 0.13</td>
<td>September: 8.16-10.13</td>
<td>month n.s.</td>
</tr>
<tr>
<td>x males (n=9) = 9.22 ± 0.11</td>
<td>9.15 ± 0.13</td>
<td>sex x month n.s.</td>
<td></td>
</tr>
<tr>
<td>Ionized Calcium, pH 7.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mm/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>x total (n=25) = 1.20 ± 0.007</td>
<td>1.23 ± 0.008</td>
<td>March: 1.14-1.26</td>
<td>sex n.s.</td>
</tr>
<tr>
<td>x females (n=14) = 1.20 ± 0.008</td>
<td>1.22 ± 0.009</td>
<td>September: 1.17-1.33</td>
<td>month *p&lt;.001</td>
</tr>
<tr>
<td>x males (n=9) = 1.20 ± 0.012</td>
<td>1.24 ± 0.014</td>
<td>sex x month n.s.</td>
<td></td>
</tr>
<tr>
<td>Serum Albumin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg/dl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>x total (n=24) = 4.36 ± 0.06</td>
<td>4.30 ± 0.07</td>
<td>March: 3.78-4.78</td>
<td>sex n.s.</td>
</tr>
<tr>
<td>x females (n=13) = 4.33 ± 0.08</td>
<td>4.21 ± 0.11</td>
<td>September: 3.77-4.71</td>
<td>month n.s.</td>
</tr>
<tr>
<td>x males (n=9) = 4.40 ± 0.08</td>
<td>4.40 ± 0.07</td>
<td>sex x month n.s.</td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Units/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>x total (n=25) = 42.06 ± 2.68</td>
<td>53.02 ± 3.28</td>
<td>March: 16.90-65.66</td>
<td>sex *p&lt;.002</td>
</tr>
<tr>
<td>x females (n=14) = 34.23 ± 2.87</td>
<td>46.60 ± 4.69</td>
<td>September: 16.13-74.11</td>
<td>month *p&lt;.001</td>
</tr>
<tr>
<td>x males (n=11) = 52.01 ± 2.80</td>
<td>61.19 ± 3.25</td>
<td>sex x month n.s.</td>
<td></td>
</tr>
<tr>
<td>Total phosphorus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg/dl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>x total (n=25) = 3.50 ± 0.07</td>
<td>3.64 ± 0.09</td>
<td>March: 3.00-4.10</td>
<td>sex *p&lt;.039</td>
</tr>
<tr>
<td>x females (n=14) = 3.61 ± 0.10</td>
<td>3.75 ± 0.13</td>
<td>September: 3.00-4.60</td>
<td>month n.s.(p&lt;.15)</td>
</tr>
<tr>
<td>x males (n=11) = 3.35 ± 0.07</td>
<td>3.49 ± 0.10</td>
<td>sex x month n.s.</td>
<td></td>
</tr>
</tbody>
</table>
3.6 Linear regression analyses of paired serum metabolites

Table 9 outlines the results of linear regression analyses performed on selected metabolite pairs. No significant regression coefficients were found for 25-OHD when regressed against ionized calcium, total calcium, 1,25-(OH)$_2$D or alkaline phosphatase activity in either March or September. Total inorganic phosphorus and ionized calcium showed an inverse but insignificant linear relationship to 1,25-(OH)$_2$D in both months. But in September a significant inverse relationship was found between total calcium and 1,25-(OH)$_2$D ($p < .05$). The same regression in the March samples was not significant.

Total serum calcium was shown to be directly related to alkaline phosphatase activity in March but not September ($p < .05$). A significant inverse relationship was found between alkaline phosphatase and total inorganic phosphorus in March but not September ($p < .05$). Finally both months showed significant direct relationships between serum albumin and total calcium (March, $p < .05$, September, $p < .01$) as well as between total calcium and ionized calcium (March, $p < .05$, September, $p < .05$).
Table 9. Significance of linear regression coefficients for selected metabolite pairs.

<table>
<thead>
<tr>
<th>Month</th>
<th>Independent variable</th>
<th>Dependent variable</th>
<th>Coefficient of Determination $R^2$</th>
<th>Slope</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>March</td>
<td>25-OHD</td>
<td>Ca++</td>
<td>0.064</td>
<td>.00146</td>
<td>1.58</td>
<td>n.s.</td>
</tr>
<tr>
<td>September</td>
<td>25-OHD</td>
<td>Ca++</td>
<td>0.0045</td>
<td>-.0003</td>
<td>0.105</td>
<td>n.s.</td>
</tr>
<tr>
<td>September</td>
<td>25-OHD</td>
<td>Alk. Phos. Act.</td>
<td>0.000</td>
<td>-.380</td>
<td>1.05</td>
<td>n.s.</td>
</tr>
<tr>
<td>March</td>
<td>25-OHD</td>
<td>1,25-(OH)$_2$D</td>
<td>0.0029</td>
<td>0.0782</td>
<td>0.055</td>
<td>n.s.</td>
</tr>
<tr>
<td>September</td>
<td>25-OHD</td>
<td>1,25-(OH)$_2$D</td>
<td>0.059</td>
<td>-.2399</td>
<td>1.20</td>
<td>n.s.</td>
</tr>
<tr>
<td>March</td>
<td>25-OHD</td>
<td>Total calcium</td>
<td>0.02</td>
<td>-.0095</td>
<td>0.46</td>
<td>n.s.</td>
</tr>
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<td>September</td>
<td>25-OHD</td>
<td>Total calcium</td>
<td>0.03</td>
<td>-.0095</td>
<td>0.78</td>
<td>n.s.</td>
</tr>
<tr>
<td>March</td>
<td>Ca++</td>
<td>1,25-(OH)$_2$D</td>
<td>0.054</td>
<td>-51.84</td>
<td>1.08</td>
<td>n.s.</td>
</tr>
<tr>
<td>September</td>
<td>Ca++</td>
<td>1,25-(OH)$_2$D</td>
<td>0.027</td>
<td>-33.17</td>
<td>0.52</td>
<td>n.s.</td>
</tr>
<tr>
<td>March</td>
<td>Total calcium</td>
<td>1,25-(OH)$_2$D</td>
<td>0.014</td>
<td>-2.42</td>
<td>0.27</td>
<td>n.s.</td>
</tr>
<tr>
<td>September</td>
<td>Total calcium</td>
<td>1,25-(OH)$_2$D</td>
<td>0.289</td>
<td>-10.17</td>
<td>7.34</td>
<td>p&lt; .05 *</td>
</tr>
<tr>
<td>March</td>
<td>Total phosphorus</td>
<td>1,25-(OH)$_2$D</td>
<td>0.063</td>
<td>-5.65</td>
<td>1.27</td>
<td>n.s.</td>
</tr>
<tr>
<td>September</td>
<td>Total phosphorus</td>
<td>1,25-(OH)$_2$D</td>
<td>0.035</td>
<td>-3.61</td>
<td>0.69</td>
<td>n.s.</td>
</tr>
<tr>
<td>March</td>
<td>Total calcium</td>
<td>Alk. Phos. Act.</td>
<td>0.251</td>
<td>17.21</td>
<td>7.37</td>
<td>p&lt; .05 *</td>
</tr>
<tr>
<td>September</td>
<td>Total calcium</td>
<td>Alk. Phos. Act.</td>
<td>0.044</td>
<td>7.62</td>
<td>1.02</td>
<td>n.s.</td>
</tr>
<tr>
<td>March</td>
<td>Alk. Phos. Act.</td>
<td>Total phosphorus</td>
<td>0.208</td>
<td>-0.011</td>
<td>6.03</td>
<td>p&lt; .05 *</td>
</tr>
<tr>
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<td>Total phosphorus</td>
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<td>-0.009</td>
<td>3.01</td>
<td>n.s.</td>
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<td>Albumin</td>
<td>Total calcium</td>
<td>0.224</td>
<td>0.69</td>
<td>6.35</td>
<td>p&lt; .05 *</td>
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<tr>
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<td>Albumin</td>
<td>Total calcium</td>
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<td>0.70</td>
<td>8.90</td>
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</tr>
<tr>
<td>March</td>
<td>Total calcium</td>
<td>Ca++</td>
<td>0.184</td>
<td>0.037</td>
<td>4.96</td>
<td>p&lt; .05 *</td>
</tr>
<tr>
<td>September</td>
<td>Total calcium</td>
<td>Ca++</td>
<td>0.193</td>
<td>0.039</td>
<td>5.27</td>
<td>p&lt; .05 *</td>
</tr>
</tbody>
</table>
Chapter 4

Discussion

4.1 Seasonal variation in 25-OHD

Seasonal variation in vitamin D status, as shown by monthly variation in the largest circulating metabolite, 25-OHD, has been demonstrated in several studies in Antarctic, subarctic, and Arctic regions (Fairney et al. 1979, Holmberg and Larsson 1980, Vik et al. 1980, Lamberg-Allardt et al. 1983, 1984, Lamberg-Allardt 1984ab, Savolainen et al. 1980) as well as in numerous lower latitude regions but no such studies to date have been conducted in subarctic or arctic latitudes in the United States. In this study of healthy Fairbanks residents we found a highly significant seasonal variation in serum 25-OHD in a group of Alaskans, which corresponded to significant measured seasonal changes in sunlight exposure. No significant rise in sunlight exposure over mid-winter values for the full group was seen until May and this corresponded to a significant rise in serum 25-OHD in June. This apparent lag in the elevation of serum
25-OHD after an increase in sun exposure is well documented and is indicative of the time period required for the metabolism of newly formed vitamin D (Parfitt et al. 1982, Mawer 1980).

The lowest average winter 25-OHD value, 23.1 ng/ml, was found in March and the peak value of 34.4 ng/ml was found in July, one month after the annual extreme value of UV light. The lowest average value is well out of the risk range for subclinical or clinical vitamin D deficiency (Haddad and Stamp 1974). Seasonal mean values are very similar to seasonally changing values found in lower and higher latitude Scandinavian countries: Denmark, Finland, Norway and Sweden (Lamberg-Allardt et al. 1983, Lamberg-Allardt 1984ab, Holmberg and Larsson 1980, Lund and Sorensen 1979, Savolainen et al. 1980, Vik et al. 1980). In the Scandinavian countries a high vitamin D status is attributed to a high vitamin D intake primarily from the consumption of large amounts of fish and fish oils. Vitamin D fortified margarines are also consumed in these countries.

Comparison of this study to lower latitude U.S. studies shows that the Alaskan 25-OHD values are predictably lower than values from healthy individuals living in the northern parts of the U.S., but differences between average yearly mean low and high values are very
similar, with a peak to trough difference of 9-15 ng/ml (Chesney et al. 1981, Haddad and Chyu 1971, Haddad and Stamp 1974, Neer 1985, Preece et al. 1974, 1975, Roginsky et al. 1974, Shepard et al. 1979, Sowers et al. 1986). Stryd et al. (1979) reported somewhat lower mean winter and summer values in Michigan than in the present study, but his study included analysis only of 25-hydroxycholecalciferol. Average spring values from this study are more than twice as high as those reported for Caucasians living in Arctic Canada (von Westarp et al., 1981), and about 65% higher than those recently reported for elder Aleuts in Alaska (Mazess et al. 1985).

Finally, these values are sometimes similar to and sometimes considerably higher than many reported values from healthy adults, for all seasons from lower latitude western European nations (Devgun et al. 1981, Ellis et al. 1977, Juttman et al. 1981, McKenna et al. 1985, Poskitt et al. 1979, Preece et al. 1974, Stamp and Round, 1974). This finding can be attributed to the much lower consumption of vitamin D-rich foods, partially due to the lack of fortification of milk and breakfast cereals and the lower consumption of fish in these countries. In most western European nations, margarine and some infant formulas are the only food items fortified, and the use of margarine is on the decline.
Seasonal variation in vitamin D intake, as well as seasonal changes in sunlight exposure, may also be influencing elevated summer D status. A study in Finland found a significantly lower D intake in a group of elderly in winter when compared to spring, summer and fall intakes (Lamberg-Allardt 1984a). In the present study, MANOVA of quarterly vitamin D intakes, when expressed as IU/kg^{0.75 dit} showed a marginally significant month effect (p < .055) detecting a rise in vitamin D intake in the spring and summer perhaps corresponding to an overall rise in food consumption with presumed increased outdoor activity and energy expenditure during those months. Quarterly caloric intake was not measured, but the % IU from fortified foods (yearly mean=47%) remained the same in both sexes over the course of the year indicating that a rise in vitamin D intake would correspond to a rise in consumption of both D-fortified and unfortified foods. There was no indication that choices of vitamin D-containing foods changed over the study year.

A final possible influence on seasonal variations in 25-OHD is the natural spring and summer increase in vitamin D content of certain foods. Researchers have shown that certain foods produced in the summer months have greater anti-rachitic value than foods grown during other seasons (Chick and Roscoe 1926). A group of elderly
in Scotland were shown to have a significant summer rise in 25-OHD even though they had no known exposure to sunlight (Devgun et al. 1981). Since vitamin D-fortified foods contributed almost 50% of the vitamin D consumed in the present study and because many animal feeds are now fortified in a similar way yearround, a seasonally changing vitamin D content in foods was probably not of major importance in affecting circulating 25-OHD levels.

4.2 Sexual difference in 25-OHD

Some studies assessing vitamin D status have noted no differences in 25-OHD levels between the sexes (McKenna et al. 1985, Kano et al. 1980). Other studies have shown that females had higher circulating 25-OHD (Mazess et al. 1985, Sedrani et al. 1983, Stamp and Round 1974), but most have found that males had higher circulating 25-OHD (Dabek 1980, Omdahl et al. 1982, Parfitt et al. 1982, Stryd et al. 1979, Stephens et al. 1982, Vik at al. 1980). In the present study a highly significant sex difference in total 25-OHD levels was found yearround with male values, on the average, 16% higher than female values. Stryd et al. (1979) in another U.S. study, found a very similar difference with males having a 13% increase over females yearround in serum 25-OHD₃.
The difference between the sexes seen in this study, can probably be explained by the significant yearround trend found for greater sunlight exposure in the males, as well as for daily vitamin D intake for males when expressed as IU/d, averaging 40% more than the female intake. Stephens et al. (1982) found similarly that Asian boys and adult males were spending more time in the sun than Asian females. No significant differences in serum 25-OHD were found between male and female adults but Asian boys had significantly higher 25-OHD than girls. In a study of Finnish children, males were found to be consuming 25% more vitamin D than females, unfortunately serum 25-OHD was not measured (Lamberg-Allardt 1984c).

When vitamin D intake was corrected to metabolic body size (IU/kg\(^{(0.75)}\).d) the sex difference was no longer statistically significant although all four quarterly mean intakes for males were greater than female intakes and averaged 26% larger for the year. Despite the statistic, it appears that males were consuming more vitamin D than females above and beyond absolute metabolic body size requirements which could be indicative of higher metabolic rates in males (Briggs and Calloway 1979) or of greater levels of physical activity leading to greater caloric intakes and thus greater vitamin D intake, resulting in higher serum 25-OHD in males. Future studies should
include assessment of both seasonal sunlight exposure and seasonal activity levels, as well as caloric intakes to study these proposed differences between the sexes.

No significant difference was seen between the sexes at the end of winter or summer in 1,25-(OH)_2D in the subsample of non-island visitors indicating that removal of 25-OHD into the most active metabolite pool due to higher estrogen levels in females, as has been suggested by Stryd et al. (1979) is not occurring. It is possible that the females, with higher body fat levels, are able to store more vitamin D and its metabolites in fat-containing tissues also leading to lower circulating 25-OHD. But it seems more plausible from this study, and from others that have found the reverse trend between the sexes, that behavioral differences in diet habits and sunlight exposure can explain the contradictory findings of sex differences in circulating 25-OHD.

4.3 Effect of D intake and sun exposure on 25-OHD

Differences between low and high vitamin D intake groups predictably affected 25-OHD levels yearlong and seemed to be the more important determinant of yearround vitamin D status than differences in sunlight exposure, which affected 25-OHD levels only during the spring and summer months. This finding is different from some
studies where sunlight exposure seemed to be the major factor influencing vitamin D status (Poskitt et al. 1979, Fraser 1981) In many of those studies average vitamin D consumption was much less than the RDA so that differences in sunlight exposure became more critical. The present results can be explained by the relatively high average vitamin D intake, slightly above the RDA and the fact that the group as a whole was composed of indoor workers who were only moderately outdoor-active, averaging an hour a day of sunlight exposure year-round. Extremes in sunlight exposure, such as those seen in housebound individuals or yearround outdoor workers were not seen in this group of volunteers. In the sub group of individuals with the lowest yearround vitamin D status (yearly mean 25-OHD levels < 20ng/ml , n=7), all were females who consumed less than the RDA for vitamin D. In fact the average consumption for this group of seven females was 134 IU/d, or 67% of the RDA.

Although Fairbanks receives sunshine yearround, monthly sums of UV radiation drop to negligible levels from November through January, averaging 2.5 % of peak summer values. During these months, there is little probable potential for photoproduction of vitamin D, even in outdoor-active individuals. Low ambient temperature during the fall and spring months may be the more
important limiter to endogenous photoproduction of vitamin D than the limit of available UV radiation as most outdoor activity requires covering all but the face and hands approximately seven months of the year. A recent study showed that there is much potential for photoproduction of vitamin D through clothing (Hutchinson and Hall 1984), but the thickness of multiple layered clothing in Alaska probably keeps this from occurring. During the summer months residents keep much of their bodies clothed because of the persistent and annoying mosquitoes and biting flies and because of this, an Alaskan summer suntan becomes one of the face and hands. In spite of this, a significant rise in serum 25-OHD was seen in the summer and differences between low, medium and high sun exposure groups were detected during some of the spring and summer months, indicating a significant endogenous production of vitamin D.

4.4 Effect of south island visits on 25-OHD

Many studies examining seasonal variation in 25-OHD have noted the effects of vacations to sunnier locations as having a positive effect on circulating 25-OHD (Juttman et al. 1981, Poskitt et al. 1979, Stephens et al. 1982). In this study, 21% of the volunteers took south island vacations, averaging two weeks sometime over the study
year but usually during the winter months. The effect of such trips on these individuals, was to markedly raise circulating 25-OHD for one to two months after their return. The group as a whole, had significantly higher circulating 25-OHD levels during the months of March and April, with a peak increase of 10 ng/ml over the non-island visit group in March. Presuming that an individual's lowest circulating vitamin D level is found in March or April, such trips could potentially have a very significant effect in improving late winter vitamin D status. Unfortunately a majority of Alaskan residents do not take such trips often.

4.5 Seasonal and sex variation in other serum metabolites and in selected regression analyses

4.5.1 1,25-(OH)₂D

Individuals with severely lowered serum calcium, phosphorus, or 25-OHD usually have elevated serum 1,25-(OH)₂D (McKenna et al. 1985, Norman 1979). In an Alaskan study, Mazess et al. (1985) found dramatically elevated serum 1,25-(OH)₂D levels in March, and low normal values of serum 25-OHD levels (mean= 16.6 ng/ml) in a group of 53 adult Aleutian Islanders from St. Paul Island, Alaska (57° N Lat). Their study showed a significant
inverse linear relationship between 1,25-(OH)_2D and % bone mineral content in females but not in males.

In this study, abnormally high 1,25-(OH)_2D values were not found for a subsample of non-island visitors in either March (end of winter) or September (end of summer). In fact values were well within U.S. norms (Chesney et al. 1981). In addition no significant inverse regressions were found between ionized calcium or total inorganic phosphorus and 1,25-(OH)_2D. A significant inverse relationship between total calcium and 1,25-(OH)_2D was found in the September but not in the March samples. In general these findings suggest that both the calcium and vitamin D status of this healthy group were not severely impaired. Conclusions are limited because of the small sample size examined in only two months of the year.

4.5.2 Ionized calcium, total calcium and phosphorus

Several studies have demonstrated seasonal shifts in calcium balance in normal individuals and found that balances were better during the summer months than during winter months (McCance and Widdowson 1943, Malm 1958). Neer et al. (1971) were able to improve calcium absorption in a group of elderly in winter by treating them with artificial (UV) light. Gupta et al. (1974) found a seasonality in hypocalcemia in Indians living in Britain.
Serum calcium levels in this group were significantly correlated with serum 25-OHD and hypocalcemic conditions found in the spring were spontaneously corrected during the summer. Vik et al. (1980) found lower serum calcium and phosphorus levels in March than during summer months, although 25-OHD levels were quite adequate during both seasons. Presumably these findings were due to photoperiod changes affecting the vitamin D status of the individuals and consequently their ability to absorb and mobilize calcium.

Serum ionized calcium (Ca++) for our group showed a slight but highly significant rise within the normal range between March and September in the subsample of yearround residents (mean = 1.20 mm/l, March; mean = 1.23 mm/l, September), indicating a possible enhancement of calcium balance by the end of summer. But because no seasonal change in serum 1,25-(OH)₂D or total calcium was seen, another explanation for the ionized calcium shift must be sought.

It is possible that the marginally significant month effect seen in vitamin D intake for the group when intakes were expressed as IU/kg⁰.⁷⁵·d, if indicative of an overall summer rise in caloric intake, could mean that summer calcium consumption was correspondingly increased. Ionized calcium can enter intestinal epithelial cells both
by simple diffusion as well by vitamin D-assisted mechanisms (Wasserman et al. 1984) thus a possible seasonal increase in dietary calcium may be affecting serum Ca++ irrespective of levels of 1,25-(OH)$_2$D. A significant positive relationship was found between total calcium and ionized calcium in both months. Future studies in Alaska should include seasonal analysis of both blood calcium and dietary calcium intakes.

4.5.3 Alkaline phosphatase

Lowered vitamin D status resulting in the deficiency disease, rickets, is often accompanied by elevations in serum alkaline phosphatase activity; but in the adult deficiency disease, osteomalacia, elevations in enzyme activity are not always seen. Two recent studies found marked elevations of alkaline phosphatase in vitamin D deficient elderly adults (McKenna et al. 1985, Lamberg-Allardt 1984 b). These elevations are thought to originate from an anomalous rise in osteoblast activity and subsequent release of skeletal alkaline phosphatase into circulation (McComb et al. 1979). In the present study no elevation of alkaline phosphatase activity was seen at the end of winter when D status was lowest, again indicating no overt disturbance of calcium metabolism.
Instead, a significant rise in enzyme activity was seen within the normal range from March to September.

Elevations in serum alkaline phosphatase activity can be caused by many differing disease states to which this healthy group was not prone. Therefore another explanation for the elevation is proposed. Increases in physical exercise have been shown to cause transient but significant increases in serum alkaline phosphatase activities (McComb et al. 1979). Moderate exercise is associated with bone accretion and increased osteoblast activity. Perhaps seasonal increases in outdoor physical activity, presumed to be higher in summer when ambient temperatures and sunshine hours are greatly increased, have resulted in higher serum alkaline phosphatase from increased osteoblast turnover. This hypothesis cannot be verified as seasonal changes in physical activity were not monitored. In addition, the isoenzyme profile of the alkaline phosphatase was not analyzed so an osteoblast (or skeletal) origin for the increase in enzyme levels and/or activity is not known.

Sex differences in serum alkaline phosphatase activity have been noted in most studies (McComb et al. 1979) and this study has similarly found enzyme activity levels significantly higher in males than females in both March and September. The cause of this sex difference has
not yet been discerned. In this study total inorganic phosphorus levels were found to be significantly higher in females than in males in both months. Inorganic phosphorus, one of the products of alkaline phosphatase activity, has been shown to inhibit the activity of this enzyme (McComb et al. 1979) therefore the elevated serum phosphorus seen in females may be having a negative effect on enzyme activity. The significant inverse regression found between total phosphorus and alkaline phosphatase activity for the group in March, supports this hypothesis. The same regression for September had a similar slope but was not statistically significant.

Lastly, an interesting positive relationship was found between alkaline phosphatase activity and total serum calcium in March but not September. Because higher serum calcium levels are required for increased osteoblast and anabolic bone building processes, one could hypothesize a skeletal origin for this increase in enzyme activity associated with individuals having higher serum calcium available for such processes. Such a hypothesis would again require an isoenzyme study to discern the origin of the presumed increased enzyme levels.
4.6 Conclusion

In conclusion, a highly significant seasonal variation in serum 25-OHD was found with an elevation above winter and spring values occurring in June one month after a significant rise in sunlight exposure. Although differences in yearly sun exposure affected serum 25-OHD in spring and summer months, differences in dietary vitamin D affected individuals more dramatically all year long. Thus exogenous intake of vitamin D should be considered to be the more important determinant of yearround vitamin D status in view of the unique limitations to endogenous photoproduction of vitamin D in Alaska. Almost 50% of the vitamin D intake came from fortified foods, and had these not been available, the average consumption for this nutrient would have been approximately one half of the RDA, or 100 IU/d. Other prominent sources for this nutrient came from fatty fish (salmon and tuna), eggs, and processed meat products.

Average monthly values from this study are slightly lower than those found in healthy adults in higher latitude U.S. studies, but higher than those recently reported from arctic Canada and Alaska. All the average monthly mean values were considered to be well above rachitic values, although a few individuals fell below 10
ng/ml during some of the winter months. The present study had very similar, if not identical values to northern European studies where consumption of vitamin D-rich foods is quite common. But these Alaskan findings are in sharp contrast to the much lower normal values reported for western and southern European nations where vitamin D consumption is markedly less.

An unexpected difference in 25-OHD was found between the sexes with males having values 16% higher than females on the average yearround. This can be adequately explained by the large significant difference in vitamin D intake (IU/d) averaging 40% more in males than females. There is also a trend for vitamin D intake, when expressed on a metabolic body weight basis, to be larger for males than females in all four quarters examined. In addition, there was a significant trend for males to spend more minutes exposed to sunshine each day than females, giving them a better chance for photoproduction of vitamin D.

All those individuals with yearly mean 25-OHD levels below 20 ng/ml were females (15 % of the study group), indicating that the difference between the sexes places females at greater risk for development of vitamin deficiency.

A considerable effect of vacations to sunnier locations was seen with a marked elevation in late winter
serum 25-OHD values above those who did not take such vacations. These trips impart a considerable elevation in vitamin D status during a time of year when vitamin D status is normally lowest.

This study of vitamin D status in healthy Fairbanks residents provides the first yearround baseline data for comparison with other Alaskan groups considered to be at greater risk for subclinical and clinical vitamin D deficiency. These findings showed that normal levels of serum calcium, phosphorus, 25-OHD, $1,25-(OH)_2D$ and alkaline phosphatase were maintained despite the extreme seasonal differences in sunlight exposure. Caution should be taken in extrapolating these findings to other Alaskan groups, as this healthy group probably represents the highest potential for adequate nutritional status at this latitude.

Seasonal shifts in circulating vitamin D metabolites, ionized calcium, and alkaline phosphatase may indicate a net seasonal shift in calcium balance and bone growth and resorption in certain individuals. Further detailed studies would be needed to test this hypothesis.

Finally, assessment of vitamin D status of groups at greater risk for vitamin D deficiency (i.e. elderly, children, mothers, darker-skinned individuals, alcoholics, and in general, lower income groups), should be carried
out in light of the lowered vitamin D status usually found for these groups at lower latitudes in the United States. Low circulating vitamin D levels coupled with typically inadequate consumption of calcium, could place these groups at greater risk for developing osteoporosis later in life.
Literature cited


Lambert-Allardt, C. 1984a. Vitamin D intake, sunlight exposure and 25-hydroxyvitamin D levels in the


VOLUNTEER QUESTIONNAIRE—VITAMIN D STUDY

NAME: _______________________
WORK PHONE #______________
HOME PHONE #______________
AGE: ________________

1) How many years have you lived in Alaska? ________

2) Are you planning any vacations in the next 12 months? _____ Where?
   fall ______________________
   winter ____________________
   spring _____________________
   summer ____________________

3) Are you taking any vitamin and/or mineral supplements? yes___ no____

4) Would you be willing to refrain from taking Vitamin D and calcium
   supplements over the course of this year? yes___ no____

5) Do you use sunlamps or tan yourself at tanning centers? yes___ no____

6) Do you eat breakfast? yes___ no____

7) Would you be willing to skip breakfast or eat very lightly the
   morning you come in to donate blood? yes___ no___ (perhaps
   bring a snack to eat after the blood has been taken...) 

8) Are you a milk drinker? yes___ no___
   What kind of milk? ______________ (brand name)
   Is it Vitamin D fortified? yes___ no___ unknown____
   How much milk do you drink per week? ______________

9) Are you in good health? yes___ no___
   comments: ____________________________________________________________

10) Do you have any history of:
    a) liver disease yes___ no___ ________________________________
    b) kidney disease yes___ no___ ________________________________
    c) intestinal disease yes___ no___ _____________________________
    d) dietary disease yes___ no___ ________________________________
    e) hormonal disease yes___ no___ ______________________________
    f) chronic disease yes___ no___ ________________________________
    g) seizure disorder yes___ no___ ________________________________

11) Are you on any sort of a special diet? yes___ no___
    If yes, what: ______________________________________________________

12) Are you taking any medications? yes___ no___
    a) prescribed: _______________________________ (BC pills?)
    b) self-selected: ____________________________ (Tums, Rolaids?)

13) Would you like a reminder card in the mail before the start of your next
    diet survey? yes___ no___

Females only:

Appendix A. Volunteer Questionnaire.
STATEMENT OF COMPLIANCE

Special Institutional Assurance in Connection
With Single Projects Involving Human Subjects

The University of Alaska will comply with the policy for the protection of human subjects participating in projects or activities supported by grants and contracts made by the Department of Health, Education and Welfare. This policy requires a review independent of the investigator or director to safeguard the rights and welfare of these subjects. An initial review of the application for a grant or contract identified as Seasonal Variations in Serum Vitamin D and Calcium Levels in a Fairbanks Community: Relation to Variations in UV Radiation and Diet submitted by this institution on behalf of Meredith G. Tallas, graduate student, Dept. of Biology, Institute of Arctic Biology, University of Alaska, Fairbanks, Alaska, 99701, (907) 474-7164 indicates that:

(1) In the opinion of this committee the risks to the rights and welfare of the subjects in this project are:

- those risks associated with venipuncture including slight pain and possible temporary discoloration at the venipuncture site
- those risks associated with collection of data on personal dietary and behavior habits of the volunteers, problem of confidentiality and anonymity when data is presented and published

The committee states that adequate safeguards against these risks have been provided as follows:

- the blood samples (15 cc.) will be drawn by an experienced technician, a phlebotomist
- sterile disposable vacutainer tubes and needles will be used
- data collected from subjects will be kept confidential and the data which is made public will not include subject names; subjects will remain anonymous

(2) In the opinion of the committee the potential benefits of this activity to the subjects outweigh any probable risks. This opinion is justified by the following reasons:

- by obtaining measurements of seasonal variations in serum 25-(OH) vitamin D and calcium, a better understanding of man's ability to maintain optimal vitamin D and Ca nutritional status during months of low light intensity

Appendix B. Human experimentation committee approval forms.
will be obtained. If evidence of low vitamin D or calcium intakes is found and affects the blood picture adversely (when compared to norms for healthy individuals at lower latitudes) then appropriate recommendations will be made to correct these nutrient deficiencies. These recommendations could lead to the overall improvement of the nutritional status of Fairbanks residents and others living at comparable or higher latitudes.

(3) In the opinion of the committee the following informed consent procedures will be adequate and appropriate:

Written consent will be obtained from each volunteer (see proposed consent forms attached). Information collected from each volunteer will be published when necessary but subjects' names will not be used. Each subject will be given a number code for the purpose of filing and tabulating data and analyzing samples.

(4) The committee agrees to arrange for a continuing exchange of information and advice between itself and the investigator or director particularly to deal with proposed changes in project or activity design or with emergent problems which may alter the investigational situation with regard to the criteria cited above. This exchange will be implemented through:

- a required committee review of any departure from approved protocol
- a report following one year from the start of the project

(5) The signatures, names, and occupations or titles of the members of the committee are listed below.

<table>
<thead>
<tr>
<th>Signature</th>
<th>Name</th>
<th>Occupation or Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signature</td>
<td>Robert Olmstead</td>
<td>Professor of Human Nutrition</td>
</tr>
<tr>
<td>Signature</td>
<td>Frederick A. Milam</td>
<td>Prof. of Human Ecol.</td>
</tr>
</tbody>
</table>

Appendix B. (cont.)
I certify that this review was carried out in accordance with the provisions of DH EW policy.

(6) Official signing for Institution

James R. Cook
Signature
Name
Professor of Medical Science
Title
University of Alaska
Institution
Fairbanks, Alaska 99701
Address
(907) 474-7080
Telephone number
Date 11 July 1983

Appendix B. (cont.)
UNIVERSITY OF ALASKA
Institute of Arctic Biology

VOLUNTEER’S CONSENT FORM

Research Project Title: Seasonal Variation in Serum Vitamin D and Calcium Levels in a Fairbanks Community: Relation to Variations in UV Radiation and Diet

Research Procedure Description: Fifteen ml. (½ fl. ounce) of blood is requested from each volunteer to be drawn by an experienced technician (a phlebotomist) from a vein in the arm. Sampling will be done at the beginning of each month, during the morning hours only beginning in September 1983 and continuing for 12 months, through August 1984. Fasting blood samples are preferred. Volunteers will be given diet history and outdoor activity charts on which to keep record of total foods consumed and outdoor activity for the three-day period before blood sampling is scheduled.

The blood samples will be analyzed for serum 25-(OH) vitamin D and total calcium and compared to calculated dietary intakes for these nutrients. Serum nutrient levels will also be compared to estimated sunlight exposure as calculated from activity charts.

Procedure Demands: 1) Blood test: the monthly venipuncture required could cause slight pain and possible temporary discoloration at the venipuncture site. 2) Diet and Outdoor Activity Records: Information collected monthly from these charts, as well as blood nutrient data will be kept with the principal investigator. Records bearing volunteer’s names will be secured in locked file cabinets accessible only to the principal investigator. Publication of the results of this study will not include volunteer’s names.

AUTHORIZATIONS

The nature and demands of the study have been clearly explained to me and I understand and accept the hazards involved. I also understand that if some unforeseen complication occurs, it too, is considered to one of the hazards of being a volunteer. Furthermore, I understand that I may withdraw from the study if I find that I am unable to continue.

Volunteer's Signature: ____________________________
Date: ____________________________

I have carefully explained the nature, demands, and foreseeable risks of the above study to the volunteer.

Investigator's Signature: ____________________________
Date: ____________________________

Volunteer Identification:

Name ____________________________ Volunteer's Assigned # ____________________________
Mailing Address ____________________________ Zip ________ Work Phone ________
Home Address ____________________________ Zip ________ Home Phone ________
Birthdate ____________________________ Age ________ Sex ________ Occupation ____________________________
Race: 1) Caucasian 2) Black 3) Eskimo 4) Indian 5) Other ____________________________

Appendix C. Volunteer Consent Form.
# Sunlight Exposure Record

Name: ____________________________

Did you go on any vacations this month?  

yes □  no □

If yes, where: ____________________________

How long: ____________________________

<table>
<thead>
<tr>
<th>Date</th>
<th>Time of Day</th>
<th>Conditions (see code # below)</th>
<th>Minutes of Exposure</th>
<th>Portion of Skin Exposed (use code # below)</th>
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**Weather Coding:**

Cloudy = 1
partly cloudy = 2
sunny = 3

**Code # for Portion of Skin Exposed to Sunlight:**

0 = no skin exposed
1 = face exposed
2 = hands & face exposed
3 = arms & hands & face exposed
4 = legs & arms & hands & face exposed
5 = sun bathing with bathing suit
6 = nude sunbathing
7 = other -- explain with description

Appendix D. Sunlight Exposure Record Form.
[Don't forget to indicate the brands of margarine, cereal, and bread you are eating if possible...thanks]

FOOD AND BEVERAGE DIARY

NAME: ____________________________________________

DATE: ____________________________________________

<table>
<thead>
<tr>
<th>TIME OF DAY</th>
<th>WHERE Eaten</th>
<th>FOOD ITEM</th>
<th>FOOD PREPARATION</th>
<th>AMOUNT EATEN</th>
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</table>

Did you take any vitamin and/or mineral supplements today? yes no
If you did, what kind did you take: ____________________________________________

Appendix E. Food and Beverage Diary Forms.