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A Spectrophotofluorometric Analysis of Serum Vitamin A Levels in a Defined Population

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A Spectrophotofluorometric Analysis of Serum
Vitamin A Levels in a Defined Population

by

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<u>Curriculum Vitae</u>

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PART I

INTRODUCTION

A. Objectives of Investigation

This study was undertaken to determine serum levels of vitamin A for a defined population of pediatric patients and of dental patients undergoing periodontal surgery. Serum vitamin A levels of infants were monitored: a) to establish a baseline value for a normal infant population, b) to compare vitamin A levels of premature infants with term infants, and c) to compare infant weights with serum vitamin A levels.

B. Historical Background

The possible role of vitamin A as reported in the liter-
ature is extremely diverse. Vitamin A is reported to affect:
vision, reproductive organs, growth and formation, and mucous
membranes. The following discussion will be limited to the
role of vitamin A as related to growth and mucous membranes.

E. V. McCollum is credited with the discovery of vitamin
A (1). McCollum (2) observed that rats maintained on diets
composed of casein, carbohydrates, lard and salt mixtures
maintained a normal growth rate from 70-120 days. Beyond
this time range, no further increase in body weight was de-
tectable. Numerous adjustments were made in the diet to
obtain further growth. It was observed that growth suspension
was prevented by the addition of an ether soluble extract from
eggs and butter. McCollum postulated that the animals de-
plete some organic complex which is indispensible for further
growth.

In 1924 it was shown that rats maintained on vitamin A
deficient diets developed atrophy of the stomach and intes-
tinal mucosa, and epithelial changes in the stomach result-
ing in hyperplasia, papilomatous growth and formation of
ulcers (3). A reduction in number of goblet cells and mu-
cous secreting capacity was shown for vitamin A deficient
animals (4). Mori (5) was the first to draw attention to
epithelial changes (drying) in the mucous membrane of larynx,
trachea and ducts of major salivary glands (parotid, sub-
maxillary, submandibular). The work was followed up in con-
siderably more detail by Wolbach and Howe (6), who performed

gross and histological examinations on albino rats that were maintained on a vitamin A deficient diet. It was shown that stratified keratinizing epithelium replaced the normal epithelium in various parts of the respiratory tract, alimentary tract, eyes, paraoccular glands and the genitourinary tract. This new epithelium was not a differentiation or change of the pre-existing cells, but a stratified layer of epithelium arising from the basal cell layer as demonstrated by foci of dark staining chromatin. Growth activity of the epithelium was not diminished, but appeared to be augmented. A few animals demonstrated epithelial growth suggestive of neoplasia as indicated by the increase in mitotic figures and alterations in connective tissue and blood vessels. It also demonstrated that young rats showed evidence of a deficiency more rapidly than adult rats, indicating a difference in vitamin A reserves with age. It was concluded that epithelium loses its specific biological function which is the secretion of mucous. A post mortem histological examination of one human vitamin A deficient subject was reported (6). An epithelial change, similar to that observed in vitamin A deficient rats, was recorded in lungs, uterus, submaxillary gland ducts and the renal pelvis.

The work of Wolbach and Howe was questioned since diets were also deficient in vitamins C and D. Goldblatt (7) clarified this problem by restricting the diet to one which was deficient in vitamin A alone. This diet induced metaplasia of columnar, cuboidal and transitional epithelium to

squamous keratinizing epithelium. Sixty-three,rats 25 to 35 days old at initiation of the experiment, were divided into three groups. Animals received diets deficient in vitamin A or vitamins A and D, while a third group received a diet complete in all respects. The length of the time on the diet varied from 10 to 20 weeks. Animals on vitamin A deficient diets or vitamins A and D deficient diets both showed evidence of metaplastic changes in the nasal passages, larynx, trachea, bronchi, lungs and tongue. Abcesses of the tongue related to vitamin A deficient diets were first reported by Sherman and Mansell (8). Post mortem examination revealed a higher incidence of infection as indicated by tongue abcesses, xeropthalmia and broncho-pneumonia. Nineteen rats on the complete diet showed no foci of abnormal squamous epithelium. Sir Edward Mellanby (9) was a major contributor to elucidating the role of vitamin A as an anti-infection agent. Infective complications were consistently the cause of death in his vitamin A deficient animals. Prior to Mellanby's work several independent investigators noted the occurrence of stone formation in the urinary tract of animals on a vitamin A deficient diet (10,11). Van Leersum suggests that this may be the result of the altered epithelial lining. Vitamin A deficiency has been correlated with epithelial changes in the respiratory tract and salivary and prostate glands (12).

Excess amounts of vitamin A have also been correlated with epithelial changes. Administration of vitamin A in excess was shown to cause the transformation of normal keratinizing tissue into mucous secreting tissue (13,14).

The classic study of Mellanby and Fell (15) demonstrated that keratinizing tissue was transformed to mucous secreting tissue by using organ cultures of embryonic chicken skin and exposing them to an excessive amount of vitamin A. This was confirmed in a later study by Rothberg (16). In the presence of a vitamin A rich medium, the transition of keratinizing epidermis to mucous-secreting epithelium was demonstrated in the embryonic chick skin organ culture. Further, it was shown that fetal bone and cartilage from the embryonic chick underwent rapid resorption when exposed to hypervitaminosis A conditions (17).

The idea that different tissues have different thresholds for vitamin A was first proposed by Parnell and Sherman (18). This was based on their finding that pseudostratified columnar epithelium reverts to a stratified squamous epithelium in vitamin A deficient rats. The concept of different tissue thresholds for vitamin A was supported by the report that normal mucous secreting epithelial tissue that has undergone a carcinogen induced metaplasia could be reversed by systemic administration of vitamin A (19). Wolf and DeLuca proposed that epithelial tissue behaves in the following manner (20). The tissue with the lowest threshold is the epithelial lining of the gastro-intestinal tract. In the presence of normal levels of vitamin A, basal cells produce mucous-secreting goblet cells. A decrease in the secreting capacity and a decrease in the number of goblet cells has been shown in vitamin A deficient rats. The epidermis was proposed to have

the highest threshold of vitamin A since it is keratin

producing under normal levels of vitamin A. Conversely it

has been shown that keratinizing epidermis will undergo a

mucous metaplasia when exposed to hypervitaminosis A condi-

tions (16).

Vitamin A in various animal model systems can influence

cytodifferentiation of epithelial tissues in two directions:

1) without the vitamin, a propensity towards keratinization of

epithelial tissue exists; 2) an excess of the vitamin stim-

ulates the chemical functions of mucous secretion (21).

Prutkin (22),using 195 male mice,placed two equal size

wounds on their dorsal surface; one wound served as a control

for each animal. Vitamin A was prepared in three different

solvents: oil, lanolin, and acetone. Thirteen groups were

used to establish the effect of the application of these

solvents with and without vitamin A as well as the effect of

frequency of doses. Gross results showed that at the end

of two weeks a single topical dose had produced no evidence

of re-epithelization while animals that had received two

doses showed a thick scab and complete epithelization.

Where three doses had been applied within the two week span,

epithelization was complete in one week. The vitamin A

applied in the lanolin or mineral oil showed greater stimu-

lation than in acetone. Vitamin A appears to influence the

frequency with which basal cells of epithelium undergo

mitosis.

DeLuca (23) proposed a mode of action for epithelial

cell differentiation. Using pair-fed rats with a vitamin A

deficient diet he demonstrated a decrease in the fucose gly-
copeptide biosynthesis using a Periodic Acid Schiff stain.
A 40% decrease in prepared antiserum to the fucose-glycopep-
tide and a decrease in the immunofluorescence material
present in the trachea, conjunctiva, intestine and bronchi
of the vitamin A deficient rats was found. This was con-
firmed biochemically by a 33% reduction of ^{3}H fucose in defi-
cient rats. It was postulated that retinol may act like
the polyisoprenols in bacteria by functioning as a transfer
agent for mono or oligosaccharide to acceptor proteins for
synthesis of glycoproteins. An intermediate may be formed
consisting of retinyl phosphate sugar. During deficiencies
of vitamin A this intermediate may not be present.

Histology on tissues from animals receiving methyl pred-
nisolone showed a complete loss of mucoid substance after
four days (24). A clinical and histological examination of
animals on methylprednisolone acetate with and without vita-
min A supplementation showed a significant ($P < .01$) decrease
in ulcer formation in the animals on a vitamin A supplemented
diet. This was attributed to the increase in mucous pro-
duction of animals on the vitamin A supplements. T. K.
Hunt (25) has demonstrated in an animal study that retarded
healing during cortisone therapy can be inhibited by
administration of vitamin A. Both systemic and topical
application were used with the former showing a greater
increase in rate of wound healing. In the absence of cor-
tisone treatment, Hunt was not able to show a significant

difference in the rate of healing. He also indicated
that wounds of those animals receiving vitamin A therapy
healed more by epithelization and that complete contraction
did not occur.

Chernov (1) monitored the serum levels of vitamin A
of severely stressed trauma patients. He selected thirty-
six stressed patients and divided them into two groups to
test the prophylactic value of vitamin A in prevention of
stress ulcers. Stress ulcers were diagnosed in patients
who had obvious upper gastrointestinal bleeding that required
multiple blood transfusions or those who had evident ulcers
at operation or autopsy. Of the thirty-six patients selec-
ted, twenty-nine had a significant decline in serum vitamin
A levels 24-72 hours after admission into the hospital. Us-
ing a fluorometric assay for serum vitamin A determinations,
fourteen of the thirty-six patients demonstrated levels below
10ug/100ml. Once the patients were placed on a high protein
diet, serum vitamin A levels returned to normal. Evidence
of stress ulcers were seen in 15 of 22 patients not treated
with vitamin A. Of fourteen patients treated with massive
doses of vitamin A (10,000-400,000 I.U.) two had upper gastro-
intestinal bleeding. The severity of the injuries of the
two groups were similar. A similar study (26) of burn
patients whose vitamin A levels were measured by a macro-
method utilizing trifluoroacetic acid, supported Chernov's (1)
initial findings. A decline in vitamin A levels was reported
as the severity of the burn index increased. This decline
was observed 48 hours post-burn and returned to normal within

the second week. Patients with upper gastrointestinal bleed-
ing exhibited serum vitamin A levels in the low range of
18ug/100ml. The accumulated evidence suggests an important
relationship between serum vitamin A levels and the integrity
of the gastrointestinal mucous membrane.

C. Rationale of Research

Necrotizing enterocolitis in the newborn can be defined as a condition in which there is diffuse or patchy necrosis of the mucosa or submucosa in the small and/or large bowel (27). The etiology is unknown. Factors such as hypoxia, sepsis, respiratory distress syndrome and dissiminated intravascular coagulation have been suggested in current causal theories (28). The diagnosis of necrotizing enterocolitis is made in 3% to 8% of all premature infants in an intensive care center (28). It is seen primarily in infants with low birthweights who have perinatal complications such as hypoxia (29). The onset of the disease is in the early weeks of life and often precipitated by enteric feedings (28,29).

The necrotic lesions are found in all parts of the alimentary tract. Microscopically the earliest recognizable lesion is coagulation necrosis of the mucosa. Submucosal hemorrhage and mucosal ulceration are evident in more advanced cases (29). In a study of 64 cases three factors were important in the development of the disease: injury to the intestinal mucosa, the presence of intestinal bacteria, and enteric feedings. Treatment is dictated by the symptoms the infant presents with at the time of examination. For an explanation of the medical and or surgical treatment the reader is referred to Santulli (29).

With the evidence strongly suggestive of a relationship between vitamin A and mucous membrane integrity, it was pro-

posed to compare the serum vitamin A levels of premature
infants with term infants. A second population, dental
patients undergoing periodontal surgery, was also proposed
for a pilot study. This second group was selected for the
following reasons: 1) epithelization is one part of the
wound healing process, 2) the oral mucous membrane as a part
of the lining of the gastrointestinal tract is physiologi-
cally similar to the intestinal mucosa, and 3) the paucity
of the literature related to vitamin A and mucous membrane
integrity in the oral cavity suggests the need for this inves-
tigation.

D. Methods of Analysis of Vitamin A

Vitamin A gives a green fluorescence when excited with ultraviolet radiation in the region of 330 nm. It has been demonstrated that continuous irradiation causes rapid and complete destruction of vitamin A (30). The fluorescence of vitamin A was first utilized by Popper (31) for histological demonstration of vitamin A in body tissues. De (30) was the first to use the fluorescence properties of vitamin A for a routine assay of biological samples. De (30) stated that the problem of a fluorescent vitamin A assay encountered previously was due to feeble fluorescence of vitamin A as compared to that of oxidizedthiamine or riboflavin and that a more sensitive fluorometer was necessary. Vitamin A values were determined by using a fluorometric, spectrometric or colorimetric analysis. A close agreement between the three analytical procedures was reported (30). Fluorescent properties of associated compounds such as cholesterol, carotene and vitamins A and D were also measured by adding the following quantities to a 10 ml. extract containing a known amount of vitamin A. Twenty-five ug. of calciferol and one hundred ug. of carotene did not fluoresce, while vitamin A and cholesterol showed a slight amount of fluorescence. It required as much as 5 mg. of these substances to produce a fluorescence equal to one I.U. of vitamin A. Ultraviolet radiation did not alter the fluorescence property of vitamin D or cholesterol, but that of vitamin E was destroyed. Irradiation of B-carotene was found to give an increase in fluorescence values up to

60-70 minutes, after which gradual destruction occurred. After irradiation, color reactions characteristic of vitamin A were not found nor was it of any beneficial effect in alleviating ocular lesions in vitamin A deficient rats .

Work performed prior to De by Fuyita and Aoyama (32) in 1951 described a quantitative assay for total vitamin A acetate in different oils. Using a visual comparator they found that carotene and vitamin D interfered with the assay. Vitamin A determination in biological samples must be critically examined from two major aspects. They are 1) adequacy of the extraction procedure, and 2) specificity and sensitivity of the analytical procedure. The solvent must extract the material in question completely and be suitable for fluorometric determination of the vitamin. Kahan (33), using many solvents for the extraction of vitamin A from blood, found that cyclohexane as a solvent yielded the highest extraction with the lowest blank reading. The partition of vitamin A into various two phase solvent systems led Kahan to study the most efficient ratio between water-ethanol and cyclohexane. After determination of an efficient extraction procedure the following areas were investigated:

1. Vitamin A levels in heparanized blood, plasma and red blood cells were determined. Only insignificant amounts of vitamin A were found in the red blood cells. It was concluded that plasma or whole blood could be used provided correction was made for the hematocrit.

2. Specificity of the procedure was determined by

destroying vitamin A by irradiation and measuring
levels of reagent fluorescence. Hemoglobin, bili-
rubin, cholesterol, and other fat soluble vitamins
did not interfere with the extraction procedure nor
with the fluorometric determination. Carotenoids,
under the conditions of the experiment, showed no
appreciable fluorescence.

3. Handling of samples in relation to light expo-
sure revealed the following: fifteen samples protec-
ted from light as much as possible during laboratory
procedures yielded a value of 5.50 ± 0.11 umol. The
same samples exposed to daylight for 4 hours and
handled under normal conditions yielded 2.76 ± 0.11
umol.

4. Vitamin A values as determined by the fluorometric
procedure were compared with $SbCl_3$ reaction and
ultraviolet absorption methods. The specificity of
the $SbCl_3$ reaction and the ultraviolet absorption
is questionable. Carotenoid pigments and oxidation
products of cholesterol have been found to interfere
with the $SbCl_3$ reaction giving erroneously high values
(34). Bieri (35) has pointed out that high carotene
levels may cause erroneously high values for vitamin
A with an ultraviolet absorption method.

Several important aspects of Kahan's work were confirmed
by another group of investigators. Hansen and Warwick (36)

using a similar extraction procedure and fluorometric deter-
mination showed the following:

1. Specificity was examined by adding normal and
high physiological concentrations of cholesterol, caro-
tene, tocopherol, and calciferol to different concen-
trations of standard solutions of vitamin A alcohol,
acetate and palmitate. No interference was noted.

2. No decrease in vitamin A serum samples was noted,
for two weeks when the samples were refrigerated.

3. It was confirmed that vitamin A acetate is the
most stable form of vitamin A and consequently is most
suited as a reference standard.

The fluorometric assay for vitamin A was reported as
simple, specific and sensitive (33). Thompson (37) inves-
tigated the reported fluorometric assays and compared the
values to those obtained using a column chromatography
technique. Using column chromatography followed by fluores-
cent analysis of the retinol fraction, the specificity of
the fluorometric procedure as reported (33,36) was inade-
quate. Petroleum ether was used to extract the lipid
soluble component of the serum. This component was placed
on an alumina column weakened with 5% water. Using petro-
leum ether as solvents the following fractions were eluted.
Figure 1 shows the excitation spectra determined on the
eluted fractions. Different excitation scales have been
used to show the fluorescent intensities. Solvents used
were 100% petroleum ether (A), 2% (B), 6%(C), 10%(D), and

20% (E) diethylether in petroleum ether. The extracts were evaporated and taken up in hexane for fluorescent analysis.

The major source of fluorescence at an excitation peak of 330 nm was retinol, which was eluted with solvents of 20% ether and 100% ether (Figures 1E and 1F). This was verified by comparison with standard retinol. A significant source of fluorescence was from the 100% petroleum ether fraction (Figure 1A). Upon further examination of this fluorescent substance, it was identified as phytofluene, a carotenoid. Phytofluene has a fluorescence maxima at 480 nm like retinol, but its excitation spectrum is different. Thompson used this difference (see Section III A) to derive a correction formula (37) which accounted for the fluorescence due to phytofluene. Thompson compared several methods as reported in the literature. Table I shows the importance of using the correction formula. The erroneously high serum vitamin A value, using a fluorometric analysis without correction of interfering substances, is discussed in further detail in section III A, B.

Figure 1 (37) - Fluorescence at 475 nm for an exci-
tation range of 250 nm to 400 nm of eluates in hexane
from an alumina column.

Chromatography of an extract of 25 ml human plasma

Fractions eluated	Identification by Fluorescence
A	phytofluene
B	retinyl esters
C	not identified
D	not identified
E	retinol
F	retinol
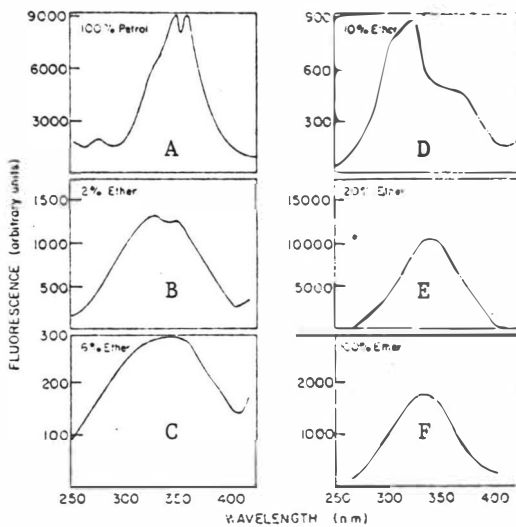
TABLE I

Comparison of Results of Correction Formula Method with

Those of Other Procedures (From Thompson et al) (37)

Procedures compared	No. of samples	Correlation coefficient	Ratio answers
Column	28	0.96	1.06+0.24
SbCl$_3$	27	0.89	1.11+0.24
SbCl$_3$, carotene correction	27	0.84	0.91+0.26
SbCl$_3$, saponified	20	0.40	1.16+0.20
SbCl$_3$, carotene correction saponified	20	0.61	0.77+0.14
Partition	16	0.95	1.18+0.14
Partition, corrected with formula	16	0.97	1.02+0.06

Mean value ± SD of answer from compared procedure ÷ answer
from correction formula.

PART II

EXPERIMENTAL

A. Assay for vitamin A

a. Reagents

All chemicals used in this study were reagent grade and used as supplied unless otherwise indicated. One normal sulfuric acid from Harleco was diluted with deionized water to make a 0.1N solution and stored at room temperature protected from light. A stock solution of quinine sulfate (Fisher Scientific) was prepared in 0.1 N sulfuric acid. Twenty-five mg. of quinine sulfate was brought to volume in a 250 ml. volumetric flask with 0.1 N sulfuric acid solution. Serial dilutions were prepared from the stock solution of quinine sulfate to a concentration of 0.01 μg/ml. The quinine sulfate stock solution and a solution containing 0.1 μg/ml of quinine sulfate were stored at room temperature and protected from light. Daily dilutions of the 0.1 μg/ml quinine solution were made to prepare the 0.01 μg/ml test solution. A 50% aqueous solution (V/V) of absolute ethanol (Commercial Solvents Corporation) was pre-pared and stored at room temperature. A nano grade hexane solvent (Mallinckrodt) was used.

b. Apparatus

All measurements in the vitamin A analysis were made on the Aminco-Bowman spectrophotofluorometer. Readings were recorded at excitation wavelengths of 330 nm and 365 nm with emission monochromator at 480 nm in quartz cells (1cm^2).

A constant temperature (25°C) compartment was maintained using a Lauda constant temperature bath. The fluorescence of 0.01 µg/ml quinine sulfate solution was determined at the same monochromator setting before and after each vitamin A sample. Glass pipettes delivering 0.2 ml t.c. were utilized for the serum transfer. A reagent dispenser (Glenco) was used for the delivery of 5.0 ml hexane to each tube. A clinical centrifuge was utilized for all centrifugation pro-cedures. A Vortex for complete mixing of the solution was utilized. Glass apparatus was used in all procedures. A Monroe calculator was used for determination of standard deviation, standard error of the mean, and the student t-test. Programma 101 (Olivetti) computer was used for vitamin A calculations.

c. Procedure

1) blood samples

Approximately five ml of blood were drawn from various patient populations : a) infant population - following clamping and cutting of the umbilical cord to separate the infant from the placenta, a volume of fetal blood remains within the placenta and cord. By unclamping the umbilical cord, this residual blood can easily be collected. This technique was used to obtain the neonatal blood samples and subsequently assayed for vitamin A. b) adult population - a standard venipuncture technique was used to obtain blood. The blood was allowed to clot for at least one hour. In all populations the sample was centrifuged at 1500 rpm

for 10 minutes. While protecting from direct light the serum was drawn off with a disposable glass pipet. The serum samples were stored at 4°C in capped and foil covered tubes.

2) preparation of serum

All procedures were performed with a minimal amount of light exposure. The serum was allowed to reach room temperature before pipetting. Glass screw capped tubes were covered with foil. To each tube 1.5 ml of a 50% (V/V) solution absolute ethanol was added. A 0.2 ml of serum was transferred to the ethanol solution. This was immediately placed on the Vortex for 5 seconds. To this tube 5.0 ml of hexane was added, followed by agitating one minute on the Vortex. The sample was centrifuged for ten minutes. Using a disposable Pasteur pipet the hexane layer was removed and the aqueous layer was discarded. A blank tube was prepared by adding 0.2 ml of the deionized water in place of the serum and performing the above procedures.

3) fluorescence determination

The Aminco-Bowman spectrophotofluorometer was used with slit openings of 3-2-5-5 in order from entrance to exit position and a sensitivity setting of 50 was used on the photometer. A 1P21 photomultiplier tube was used for all determinations. Excitation wavelengths of 330 nm and 365 nm with an emission wavelength of 480 nm were used to determine the fluorescence in the hexane layer. The fluorescence is relative % transmission which is the meter multiplier setting on the photometer x % transmission read

from the photometer. The meter multiplier was set at 0.1 for the quinine sulfate readings and adjusted for serum sample readings. A solution was prepared daily containing .01 μg/ml of quinine sulfate, which was used as a standard. The 0.01 μg/ml of quinine sulfate was read before and after each sample reading at 330 nm and 365 nm. Readings were recorded at its maximum excitation and emission wavelengths which were 350 nm and 450 nm respectively. If there was a discrepancy between the fluorescence of the quinine sul-fate reading before and after the sample determination the reading of the quinine and serum were repeated.

The calculation of vitamin A μg/100 ml serum is described in section III A.

B. Preparation of Phytofluene and Retinol for Calibration
    of Aminco-Bowman Spectrophotofluorometer

    1. Isolation of Phytofluene

        a. Reagents

            1. Hexane nanograde (Mallinckrodt)

            2. Methanol (Fisher Certified ACS)

            3. 400 grams tomatoes (fresh)

            4. Chromatographic Alumina Basic - WB-2 (Sigma)

            5. Diethyl ether, anhydrous (Fisher Reagent)

        b. Apparatus

            1. Waring blender

            2. Screw cap tubes (15 ml) with caps lined with foil

            3. Chromatography columns (8cm x 2 cm), glass

            4. Clinical centrifuge

            5. Flash evaporator (Rinco Instrument Co.)

            6. Whatman No. 1 filter paper

        c. Procedure

            1. Preparation of tomatoes

                The tomatoes were washed and cored. Four

                hundred grams of tomatoes were homogenized

                in the blender with 400 ml of methanol. The

                resulting mixture was filtered through Whatman

                No. 1 filter paper with suction. The solid

                residue was rinsed two times with 100 ml of

                methanol. The methanol extract was discarded.

                To the solid residue, 400 ml of hexane was

                added and the mixture placed on a magnetic

                stirrer for 30 minutes. The solution was

filtered and the hexane extract evaporated
under vacuum while being protected from light.
The resulting concentrate was protected from
light and refrigerated at 4°C.

2. Isolation of Phytofluene

A column of basic alumina with 2% water was
prepared. The hexane extract was placed on the
column and eluted with hexane. The phytofluene
was located by using an ultraviolet lamp (366 nm)
at a maximum distance for a brief period of
time. The phytofluene moved in front of the
colored carotenoid bands. Using a 2% diethyl
ether in hexane the phytofluene was completely
eluted. Elution was stopped when the colored
bands reached the bottom of the column. The
fraction was evaporated under vacuum. A stream
of nitrogen was used to reduce the fraction to
about 3 ml.

3. Determination of Phytofluene

The excitation spectra from 300 nm to 400 nm
was determined at an emission wavelength of
480 nm to assure the presence of phytofluene.
From the small volume of hexane containing
phytofluene, 0.1 ml was diluted with 5.0 ml
of hexane. The ratio of fluorescence at exci-
tation wavelengths of 365 to 330 at an emission
wavelength of 480 nm was determined. From

this phytofluene solution, 1.0 ml was brought
to volume in a 5.0 ml volumetric flask with
hexane. Fluorescence readings were recorded
for the diluted solution at 365 nm and 330 nm.
A ratio was calculated from fluorescent deter-
minations at 365 nm to 330 nm.

2. Isolation of Retinol

   a. Reagents

      1. Retinyl Acetate (Sigma)

      2. Absolute ethanol (Commercial Solvents Corporation)

      3. KOH pellets

      4. Hexane (Mallinckrodt)

      5. Diethyl ether anhydrous (Fisher Reagent)

   b. Apparatus

      1. Gilford Recording Spectrophotometer

      2. Thin layer chromatography - Silica-gel (Quantum Ind.)

      3. Aminco-Bowman Spectrophotofluorometer

   c. Procedure

      1. Preparation of Retinol

Retinyl Acetate (5mg) was placed in a 15 ml
screw cap tube. After addition of 1.0 ml of
H_2O, 1.0 ml of absolute ethanol and two KOH
pellets, the tube was placed in a boiling
water bath for 20 minutes with the cap off.
The tube was allowed to cool for 10 minutes.
At this time 1.0 ml of H_2O and 5.0 ml of hexane
were added and the tube was vigorously shaken.

The hexane layer was removed and evaporated under a stream of nitrogen to approximately 1 ml.

2. Retinol determination

From the hexane solution 0.25 ml was streaked on a thin layer chromatography plate. The solvent used was a 80% hexane, 20% ether solution. Retinyl acetate was also streaked on the plate as a comparison. Location of the retinol was achieved by visualization using a long wave ultraviolet lamp. Rf values were determined for retinol and the retinol band was scraped from the plate and extracted into hexane. The resulting retinol extract was diluted until an optical density at 325 nm was 0.400. A twenty-fold dilution was made by taking 0.25 ml and bringing it to volume with hexane in a 5.0 ml volumetric flask. The concentration of retinol was determined using equation 1.

$$\text{Retinol } \mu g/ml = \frac{\text{optical density x } 10^3}{1830 \text{ x } 2} \qquad \text{equation 1}$$

where

($E_{1\%}^{1cm}$ is 1830) (38)

PART III

RESULTS AND DISCUSSION

A. <u>Calculation of Vitamin A</u>

Phytofluene was isolated from fresh tomatoes as des-
cribed in section II B.   Figure 2 is the excitation spectrum
of the carotenoid phytofluene at a fluorescence emission
of 480 nm.   Phytofluene has twin excitation peaks at 355 nm
and 375 nm with a fluorescence emission at 480 nm.   The
ratio of fluorescence at excitation wavelengths of 365 nm
to 330 nm was 1.97.   The phytofluene was diluted and the
ratio of fluorescence at 480 nm determined at excitation
wavelengths of 365 nm to 330 nm.   A proportional decrease
in fluorescence equal to the dilutional factor is evident
from the data in table II.   Thus the phytofluene was at a
concentration range that a linear relationship existed be-
tween fluorescence and concentration.   If the solution is
too concentrated a self-quenching phenomena occurs (39).
The phytofluene fluorescent ratio will be used in the
correction formula for serum vitamin A determination.

The saponified retinyl acetate was separated on thin
layer chromatography as described in section II C.   The Rf
value for retinol was 0.18.   The retinol was extracted from a
thin layer chromatographic plate with hexane and the
solution diluted to an optical density of 0.401 in a Gilford spec-
trophotometer at 325 nm.   The concentration of the retinol
was 0.109 μg/ml as determined using an extinction coeffi-
cient of 1830 as described in section II.

Figure 2 - Fluorescence (480 nm) of phytofluene[1] (-0-0-)
at an excitation range of 300 - 400 nm. The reported reti-
nol spectra (38) (-----) is shown to illustrate the different
excitation peaks of the two fluorescent substances.

[1] (Phytofluene extracted from fresh tomatoes (Sec. II B)).
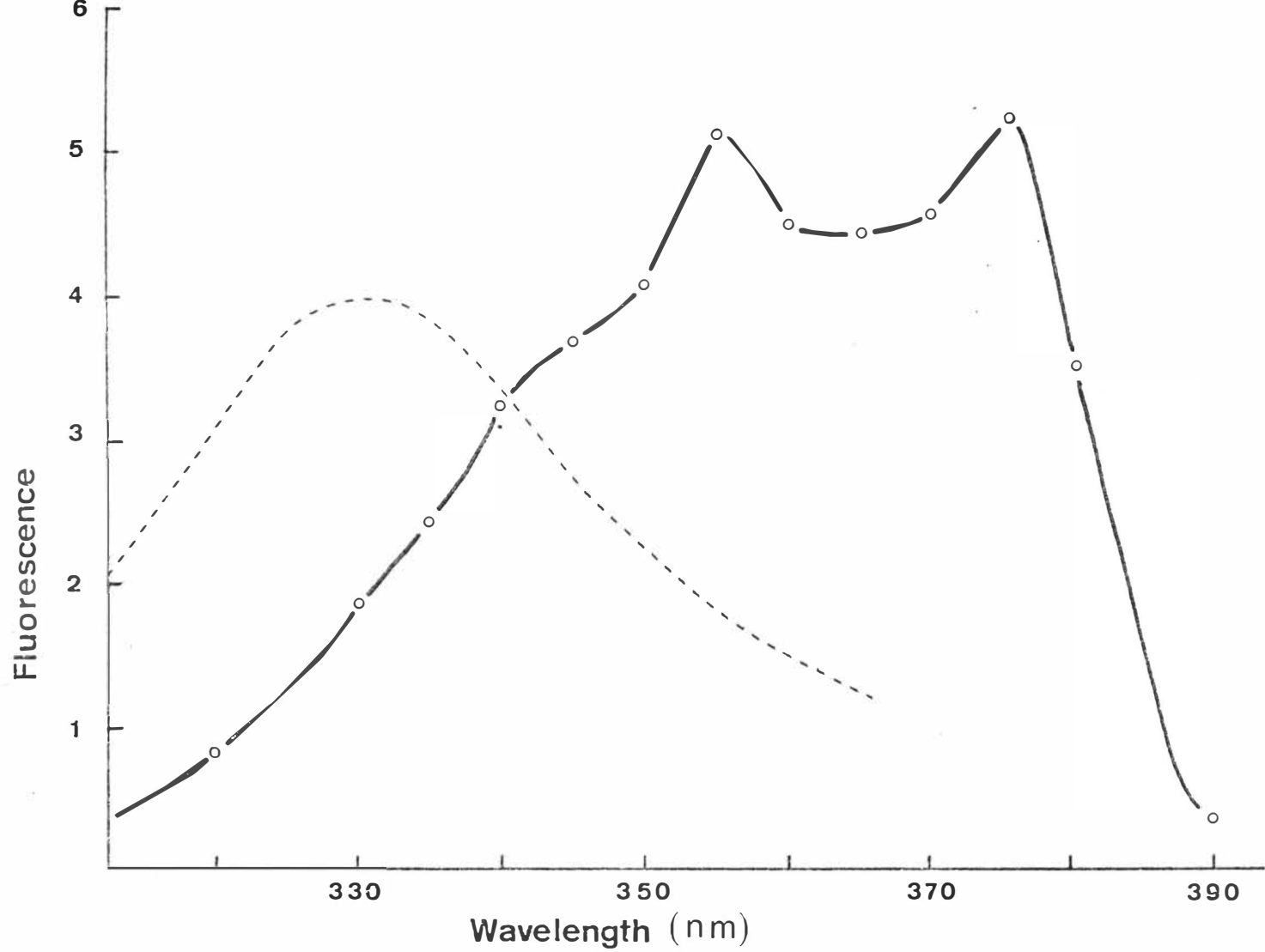
TABLE II

Ratio of Phytofluene Fluorescence at 480 nm with Excitation

at 365 nm to 330 nm

Phytofluene	Ratio	Ratio
Concentrated Phytofluene (tomato extract)	$\frac{8.28}{4.21} =$	1.97
Diluted Phytofluene[1]	$\frac{1.52}{0.801} =$	1.89

[1]Diluted Phytofluene - 1 ml of concentrated phytofluene
  brought to volume with hexane in a 5.0ml volumetric flask.

The fluorescence at 480 nm for excitation at 330 nm of a
1:20 retinol dilution in hexane was 1.15 relative % trans-
mission minus the blank fluorescence at excitation 330 nm.
A quinine sulfate (.01 μg/ml) standard reading at excita-
tion 330 nm. was 3.65 relative % transmission. A ratio of
retinol fluorescence at excitation wavelengths of 365 nm
to 330 nm was 0.42. Figure 2 illustrates a typical exci-
tation spectrum of retinol at an emission fluorescence of
480 nm.

Equation 2 represents the correction formula used
in analysis of serum vitamin A. Derivation of the formula
was previously reported by Thompson et al (37,38).

equation 2

$$\text{Retinol concentration in serum } (\mu g/100 \text{ ml}) = \left[\left(\frac{P}{P-A}\left(\frac{Q}{q}x\right)\right) - \left(\frac{1}{P-A}\left(\frac{Q_1}{q_1}y\right)\right)\right]\left(\frac{S}{R}\right)(2500)$$

where: (fluorescence at 480 nm for all determinations)

A is the ratio of retinol fluoresence at excitation 365 nm
   to excitation 330 nm

P is the ratio of phytofluene fluorescence at excitation 365
   nm to that at excitation 330 nm

Q is quinine (.01 μg/ml) fluorescence at excitation 330 nm
   at the time of calibration

q is quinine (0.01 μg/ml) fluorescence at excitation 330 nm
   at the time of serum analysis

Q_1 is quinine (0.01 μg/ml) fluorescence at excitation 365
   nm at the time of calibration

q_1 is quinine (0.01 μg/ml) fluorescence at excitation 365
   nm at the time of serum analysis

y is serum fluorescence at excitation 365 nm minus blank
   at excitation 365 nm

x is serum fluorescence at excitation 330 nm minus blank
   at excitation 330 nm

S is the concentration (μg/ml) of a standard retinol solution in hexane

R is the fluorescence of the standard retinol solution in hexane at 330 nm

The Aminco-Bowman Spectrophotofluorometer was calibrated by determining the phytofluene and retinol fluorescence ratios on the same day. At the same time fluorescence of a quinine standard (0.01 μg/ml) was determined and S/R computed. The formula reduced to equation 3 after the constants (R,A,Q,Q_1, S,R) were determined.

(equation 3)

$$\text{Vitamin A } \mu\text{g/100ml serum} = \left[1.27 \left(\frac{(3.65)x}{q330} \right) - \left(0.646 \frac{(4.75)y}{q365} \right) \right] (237.2)$$

The formula (eq.2) with the determined constants was placed on an Olivetti program card, so that only four experimental points were required to give the serum vitamin A in μg/100ml of serum. The data on the hexane extract for each sample required was the relative % transmission minus blank at excitation 330 nm and 365 nm, (fluorescence 480 nm) which are the x and y of equation 3. The other two experimental data points are the relative transmission minus blank for a 0.01 μg/ml quinine sulfate solution at excitation of 330 nm and 365 nm at emission monochromator setting of 480 nm. The quinine sulfate standard was deter-mined before and after each serum extract in order to correct for any instrumental variation. This correction is tedious and could be overcome by using a more sophisticated spectro-fluorometer which would give a constant exciting radiation. This instrumental modification is commercially available (Ratio Recording Accessory, Aminco-Bowman, J4-8912, $3,290)

and is strongly recommended to increase the total number of samples that can be determined daily.

Vitamin A is present in the serum in the retinol (Fig. 3) form (40). Vitamin A is stored in the liver and transported by a carrier protein which was isolated by Goodman in 1968 (41). The retinol binding protein is bound to retinol in a 1:1 ratio and transported in the blood in a complex with albumin (42). Before a fluorescent analysis of serum vitamin A was possible it was necessary to separate it from the protein complex. An aqueous ethanol solution was used to precipitate the serum protein. Kahan (33) reported a two solvent system using 80% aqueous ethanol solution (v/v ethanol) and cyclohexane. Thompson (37) found retinol was equally soluble in the aqueous and organic solvents using 80% ethanol and either cyclohexane or hexane as the lipid solvent. It was determined (37) that a 50% ethanol solution as the aqueous phase and hexane as the organic phase provided an efficient extraction of retinol.

On repetative sampling of serum it was found that there was some problem in precision of determination. This was found to be at the level of serum transfer. The procedure of serum transfer was examined. Table III represents a triplicate sample using a Sigma, calibrated and duo pipette (to contain) glass pipette. Serums containing normal and low vitamin A concentrations were analyzed using a duo pi-pette. The S.E.M.(standard error of the mean) for both concentra-

Figure 3 - (40) The structural formula of vitamin A$_1$

(Retinol)
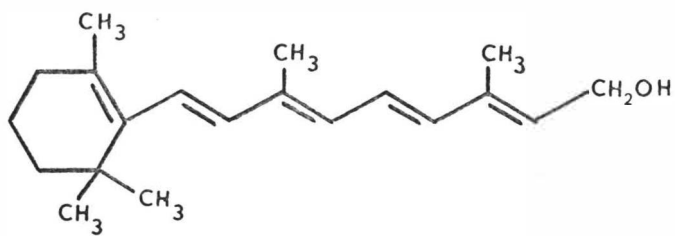
TABLE III

THREE METHODS OF SERUM TRANSFER

METHOD	MEAN[1]	S.D.	S.E.M.
Sigma	37.4	$\pm$2.8	$\pm$1.60
Calibrated Pipette	25.8	$\pm$1.68	$\pm$0.97
Duo Pipette	36.6	$\pm$0.75	$\pm$0.43
Duo Pipette	14.5	$\pm$0.42	$\pm$0.24

[1] µg of vitamin A/100 ml of serum using the correction formula shown in equation 3. Triplicate determinations were performed on all four samples.

tions were in good agreement (Table III). The duo pipette
was used in all serum transfers.

Table IV shows a comparison of serum vitamin A for
five values of vitamin A from 10.5 to 74.8 μg/100 ml using
the correction formula compared to using only the fluores-
cence at 480 nm at excitation 330 nm. Note the values
differ from 11.9% to 54.2%. Thompson et al (43) have shown
that the serum vitamin A differs by as much as 200% from
the true vitamin A levels if the correction formula were
not used.

The correction formula allows for a) a reliable
analysis of serum vitamin A in μg/100 ml, b) a correction
for day to day instrument variation by determining fluores-
cence of a 0.01 μg/ml solution of quinine sulfate, c)
correction of interfering fluorescence primarily from the
carotenoid phytofluene. Sampling techniques as well as
previously shown extraction procedures allow for the routine
analysis of serum vitamin A.

TABLE IV

Spectrophotofluorometric Analysis of Serum Vitamin A (μg/100ml) with and without
the Correction Formula
(Representative Values)

with correction formula	without correction formula	% difference[1]
10.5	19.4	84.8
26.8	33.2	23.8
44.3	68.3	54.2
68.3	76.4	11.9
74.8	91.3	22.1

[1] % difference = $\left(\dfrac{\text{serum vitamin A without correction formula} - \text{serum vitamin A with correction formula}}{\text{serum vitamin A with correction formula}} \right)$ x 100

B.   Serum Vitamin A - Normal Population

Vitamin A determinations were made on 22 faculty and students at the Medical College of Virginia.   No attempt was made to categorize according to age, sex, or other characteristics.   The mean serum vitamin A level was 60.2 μg/100 ml serum.   Table V shows the reported serum vitamin A levels of various investigators and the method of analysis used.   Warwick (36) (Table V #3) used a fluorometric analysis without correction factors.   Thompson (43) cites this as the obvious cause for the high serum vitamin A values reported.   The correction for phytofluene ranged from the equivalent of 5.8 to 54.3 ug vitamin A/100 ml. The mean correction was 29.9μg/100 ml.   This corresponds closely to the reported error (34.9 μg/100ml of the fluorometric technique without a correction formula or column separation (44).

Gary (44) compared vitamin A determinations of serum with and without chromatographic separation (Table V #4, #5).   An unknown substance was eluted on a silicic acid column that had fluorescent properties similar to vitamin A.   The addition of the fluorescence of the unknown compound and retinol resulted in a value that paralleled the fluorescent value of the serum that was not separated chromatographically.   Gary (44) and Thompson (37) have identified an interfering fluorescence that must be accounted for in the fluorometric determination of serum vitamin A levels.   This substance has been identified as phytofluene(37)

TABLE V

SERUM VITAMIN A مµg/100ml SERUM

Ref.	Mean	S.D.	S.E.M.	No. of Samples	Method
1. Current Data	60.2	±16.8	3.6	22	Spectrophotofluorometric analysis with correction formula
2. Brandt, Blievernicht Guyer (45)	50.4	±15.5	2.0	62	Spectrophotofluorometric analysis with correction formula
3. Warwick Hansen (36)	133	±42.8	5.9	53	Fluorometric Analysis
4. Gary (44)	37.2	±11.4	1.7	48(pre-school children)	Silicic acid column and fluorometric analysis
5. Gary (44)	75.1	±35.2	5.1	48(pre-school children)	Fluorometric analysis

which is corrected in the equation 2 (37) by determining
fluorescent (480 nm) values of a serum sample at excitation
wavelengths of 330 nm and 365 nm. In this report using a
fluorescent analysis with the correction formula a mean value
of 60.2 µg/100ml on 22 samples was recorded. A value of
50.4 µg/100ml (Table V) was reported using the same method
of analysis (45). Examination of the standard deviation
and standard error of the mean shows the values are in
agreement.

C. Analysis of Serum Vitamin A of Pediatric Patients

A sample population of 54 infants with a gestation
period of 36 weeks or more had a mean serum vitamin A value
of 22.5 µg/100 ml (Table VI). During serum analysis the
gestation period of the infants was not known by the investi-
gator. This value compares favorably with a reported mean(46)
vitamin A level of 23.6 µg/100ml of serum in fetal cord
blood. Samples from a population of eight premature infants
with a gestational period of less then 36 weeks demonstrated
a mean vitamin A serum level of 10.5 µg/100ml. A two tailed
Student's t-test revealed a significant difference of vitamin
A levels between these two populations (Table VI). For
this investigation, premature infants were limited to those
having a gestational period of less than 36 weeks to allow
for potential error in determining the length of gestation.
Length of gestation was determined by neurological and
physical development of the infant. Examination of the
mean weight of each group (Table VII) shows a mean of
1475 grams for the premature infants and a mean of
3393 grams for the term infants. The weight of the premature
and term infants is in agreement with reported values (47).A two
tailed Student's t-test reveals a significant (P<001)
weight difference between the two groups. Table VII gives
the serum protein values using a biuret analysis (48). A
mean value for term infants was 6.6 grams/100ml, while
premature infants had a serum protein of 5.0/100 ml.
Vitamin A per gram of serum protein for term infants was

TABLE VI

μg Vitamin A/100 ml Serum of Term and Premature Infants

Group	Number	Mean	S.D.	SEM	T-Value	two-tailed t-test P
Term (>36 wks.)	54	22.5	±7.1	±0.96		
					4.744	<.001
Premature (<36 wks.)	8	10.5	±2.2	±0.78		

TABLE VIII

Weight (gm.) and Serum Protein (gm/100ml) of Pediatric Groups

	#Samples	Mean wgt-gm	S.D.[2]	S.E.M.[3]	P Value	Mean Serum Protein (gm/100ml)	S.D.	S.E.M.	P Value
Infants of 36 week gestation period or more	54	3393	+428	58.8		6.6	+.79	.33	
					<.001				<.001
Premature Infants[1]	8	1475	+458	161.9		5.0	+.94	.11	

[1]Premature infants – a gestational period of less than 36 weeks

[2]S.D. – Standard Deviation

[3]S.E.M. – Standard Error of the Mean

3.3 μg vitamin A/gram protein/100 ml serum while premature
infants had a value of 2.10 μg vitamin A/gram protein/100 ml
serum. The level of vitamin A per gram of body weight was
.0071 μg of vitamin A/gram body weight for premature infants
and .0066 μg of vitamin A/gram of body weight for term infants.
These values give us different parameters to compare the
vitamin A levels of the two groups. It is interesting to
note that comparing vitamin A levels of the two groups on the
basis of serum protein there is a fifty-seven percent dif-
ference. Vitamin A levels of the two groups on the basis of
per gram of body weight results in very similar values. The
importance of this is not known at this time.

Figure 4 shows the serum vitamin A levels of infants
of different lengths of gestation. A general trend of
increasing vitamin A levels with length of gestation is
evident.

The low serum vitamin A levels in premature infants
suggests a predisposing factor for the increased incidence
of necrotizing enterocolitis in premature infants. The
incidence of necrotizing enterocolitis is greatest in pre-
mature infants who have undergone a stressful period at
birth (27,28). Recent reports (1,26) indicate serum vitamin A
levels decline in the stressed adult individual. A de-
crease in the incidence of gastrointestinal ulceration of
these stressed individuals occurs when administered thera-
peutic doses of vitamin A (1). It may be recalled that
one of the early signs of necrotizing enterocolitis is
ulceration of the intestinal mucosa. With this fact in

Figure 4 - Serum vitamin A levels of infants with different
lengths of gestation. Numbers in parenthesises represent
the number of samples.

Each bar represents the mean value for a group of two
or more infants of a specific length of gestation. The
standard error of the mean for each group is reported (I).
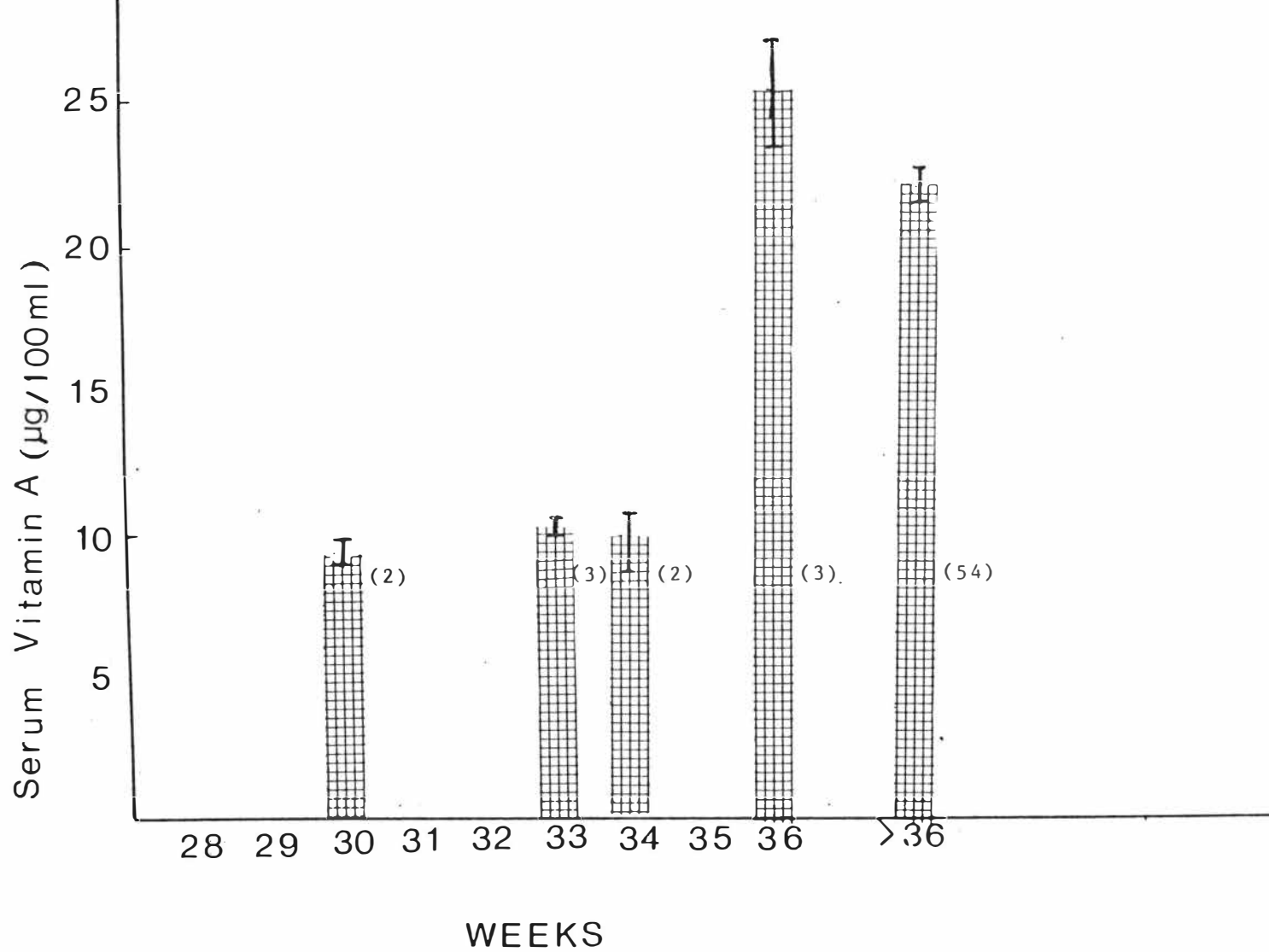
mind the therapeutic administration of vitamin A to stressed premature infants may be a beneficial prophylactic measure.

D.   Serum Vitamin A Levels of Infants and Mothers

Serum vitamin A levels of eight infants compared to their corresponding mothers by a the Student's t-test shows a significant difference ($P < .005$). The mean value of each group was 19.3 μg/100ml and 40.7 μg/100ml respectively (Table III). The group consisted of four premature infants and four term infants. Figure 5 shows the weight of each infant and the serum vitamin A level of the mother. Both premature and term groups are suggestive of a decreasing infant weight with a decreasing serum vitamin A of the mother. The small sample population prohibits further speculation. Further investigation is necessary to evalute serum vitamin A levels of the mother and its relationship to weight and serum vitamin A of the infants. A scatter plot would help determine the existence of a positive correlation of 1) infant weight vs. serum vitamin A levels of the mother and 2) serum vitamin A levels of the infant vs. serum vitamin A of the mothers.

If a relationship were determined it would be of benefit for establishing an optimal range of maternal serum vitamin A levels during pregnancy for an optimal infant level. Caution must be taken not to confuse a positive correlation between two variables with a casual relationship. The correlation may be fortuitous or a variable may exist that has not been identified.

TABLE VIII

Serum Vitamin A (μg/100ml) Levels of Infant vs Mother

	No. of Samples	Mean[1]	Standard Deviation	Standard Error Mean	t value	P value
Mothers	8	40.7	14.6	5.2	3.8	<.005
Infants	8	19.3	6.9	2.4		

[1] μg Vitamin A/100 ml serum

Figure 5   - Premature infant weights 0——0 compared to serum vitamin A levels of the mother.   Term infant weights x——x compared to vitamin A levels of the mother. Each point represents one mother and her infant.
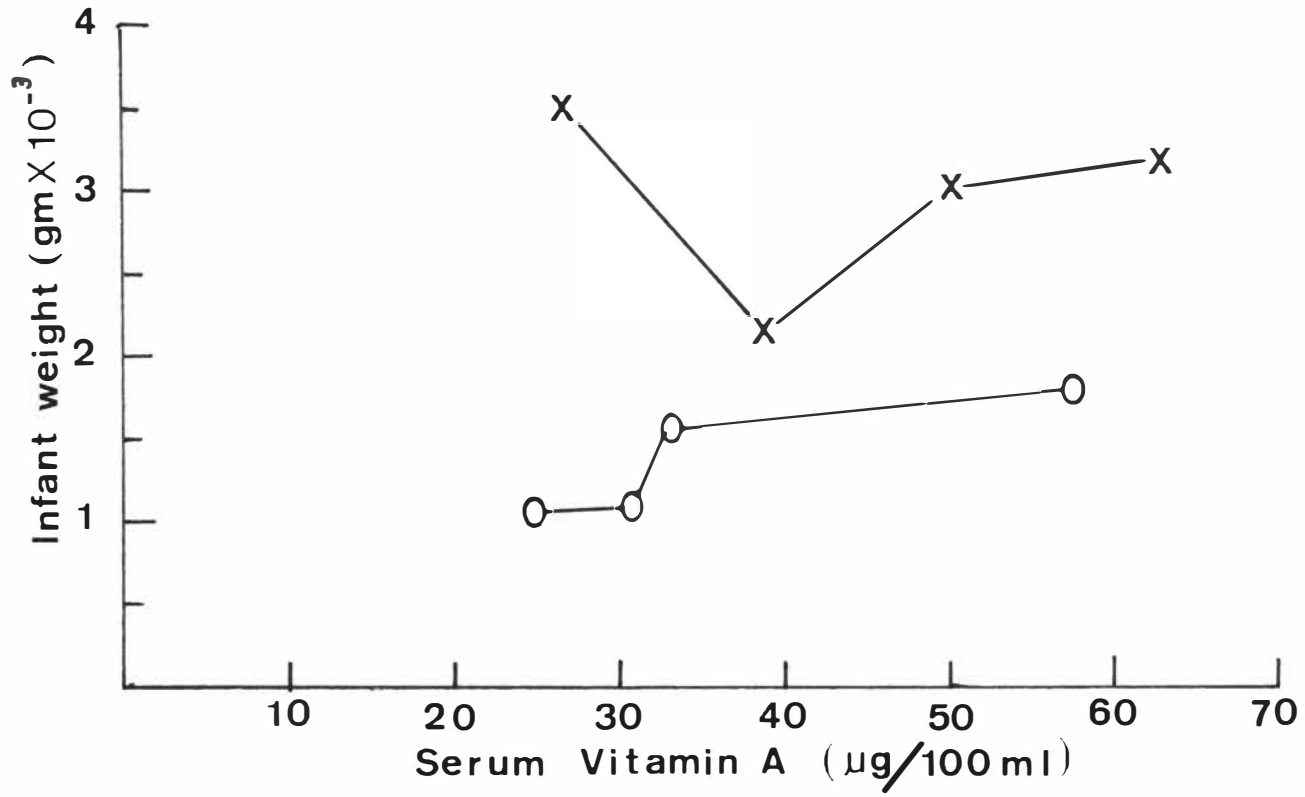
E. Serum Vitamin A Levels of Dental Patients before and
   and after Periodontal Surgery

Although the study of serum vitamin A levels of
dental patients undergoing periodontal surgery is incom-
plete the results to date and suggestions for a future
project will be discussed.

Figure 6 shows the serum vitamin A levels of patients
on the day of surgery and several days following.
Patients A and C had two surgical procedures in a four week
time period. Patients B and D each had one surgical
procedure. Patients A and C both demonstrated a decline
in their serum vitamin level. Patient A had an initial
vitamin A level of 76 µg/100ml of serum. After two weeks
which included two quadrants of periodontal surgery the
serum vitamin A level was 43 µg/100 ml. Patient C had
an initial serum vitamin level of 38 µg/100 ml of serum.
After three weeks which included two quadrants of perio-
dontal surgery the vitamin A level was 27 µg/100 ml. An
interesting observation is patient D with an initial
triplicate vitamin A determination below 10 µg/100ml. on
the day of surgery. Patient D presented 3 days later with
an elevated temperature and post-operative complication.
The serum vitamin A had increased to 23.4 µg/100ml. Patient
D was quite significant in view of the fact she was in
her mid-twenties and suffered from an advanced case of
periodontitis with a history of periodontosis as an adoles-
cent. A complete medical exam showed all systems within

Figure 6 - Serum vitamin A levels of four (A,B,C,D)
dental patients at day (O) of periodontal surgery and
intervals after surgery (X).
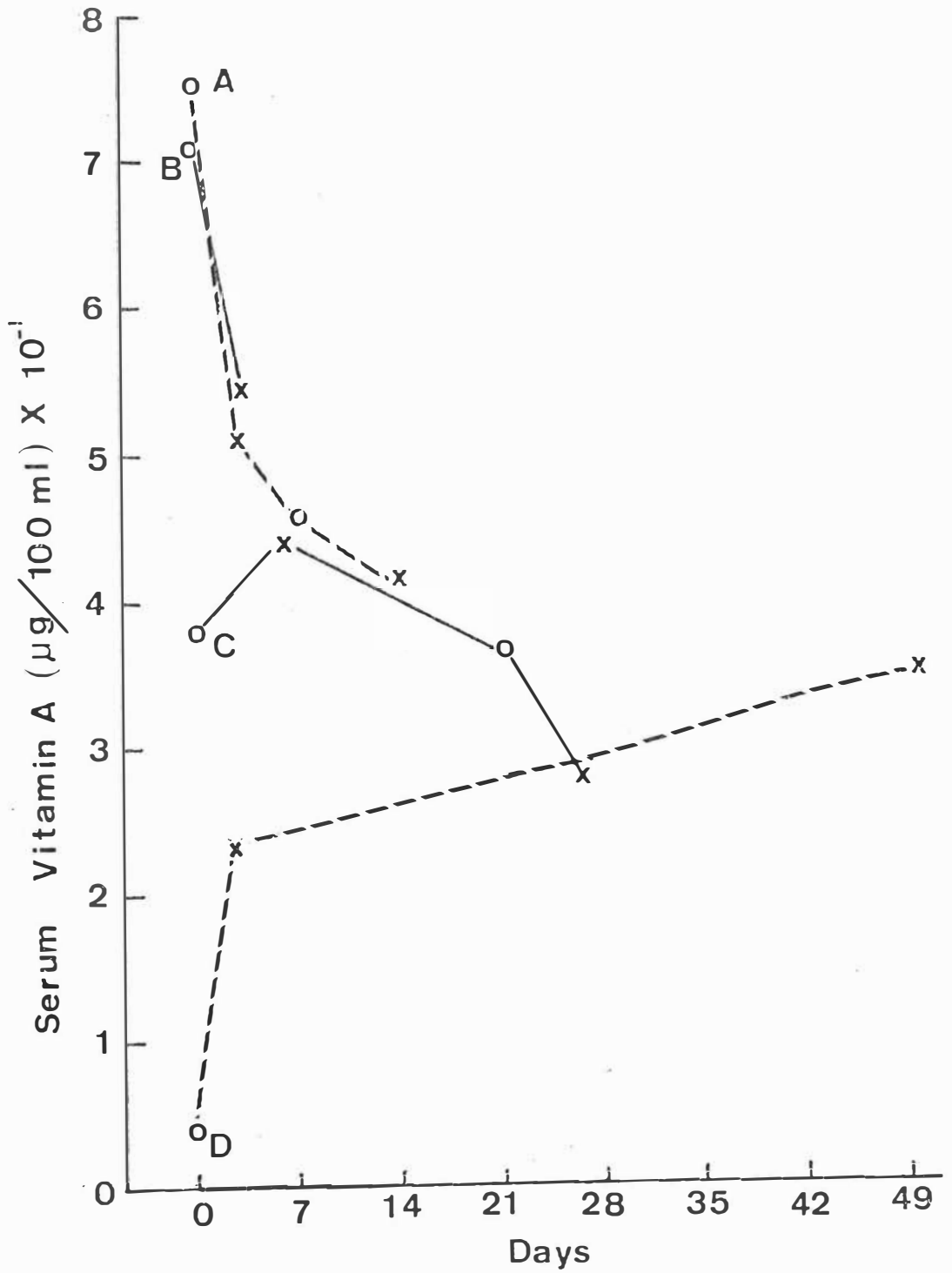
normal limits. Serum vitamin A level of patient D 49 days
after the initial surgery was 35 µg/100ml. Lack of a
control group and an inadequate baseline vitamin A level
for each individual prohibits speculation on the signifi-
cance of the results.

Problems encountered in this study will be discussed and
possible solutions suggested. The subject prior to perio-
dontal surgery undergoes an initial phase of therapy to
establish a gingival environment that would promote the
healing process (49). The time required for initial
therapy procedures such as scaling and root planing may
be from six weeks to many months. During this time period
it is recommended to obtain multiple blood samples to
establish a reliable baseline for each individual. Control
samples should be collected at the same interval as the
study group for comparison. It is recommended to provide
a financial incentive as a motivational factor for partici-
pation. A dietary history should be included both for test
and control groups. The absence of any gastrointestinal
disorders in participants should be considred since this
may affect the absorption of vitamin A or its precursors.
Further research is necessary to determine if a significant
relationship exists between serum vitamin A levels, perio-
dontal disease, and on healing following periodontal surgery.

PART IV

SUMMARY

The objectives of this thesis were to examine serum
vitamin A levels in two defined populations. The method
of analysis was a spectrophotofluorometric technique with
a correction formula to account for interfering fluores-
cence from an identified carotenoid phytofluene (37).

The first population were infants at time of delivery.
Infants were divided into two groups depending upon length
of gestation. A premature group was identified as having
a length of gestation of less than 36 weeks. Term infants
were defined as 36 weeks or greater. The mean value of
serum vitamin A of eight premature infants was 10.5
μg/100ml (S.E.M.$\pm$.78). The mean value of 54 term infants was
22.5 μg/100ml (S.E.M.$\pm$.96). Comparing these two groups
results in a p value of < 0.001. The increased incidence
of necrotizing entercolitis in premature infants, and the
fact that vitamin A is important for the maintenance of
a functional mucous membrane (28,20)gives significance to
these findings. It is suggested in this thesis that the
prophylactic administration of vitamin A to premature in-
fants may be beneficial. The comparison of serum vitamin
A levels of eight mothers and their infants resulted in
a mean of 40.7 and 19.3 μg/100ml serum respectively.
Statistical analysis revealed a p value of< 0.005.

The second population studied were patients undergoing
periodontal surgery. The purpose was to monitor serum

vitamin A levels before and after surgery. The results are inconclusive due to the small number of samples. In this thesis the results are discussed and recommendations are made for future projects in this area.

PART V

BIBLIOGRAPHY

1. Chernov, M.S.,Hale, H., and Wood, M. "Prevention of
    stress ulcers", American Journal of Surgery, 122,
    674-677 (1971).

2. McCollum, E. V., and Davis, M., "Necessity of lipins
    for growth", Journal of Biological Chemistry, 15,
    167-173 (1913).

3. Pappenheimer, A.M., and Larimou, L.D., "The occurrence
    of gastric lesions in rats and their relation to
    dietary deficiency and hair ingestion", Journal of
    Experimental Medicine, 40, 719-732 (1924).

4. Manville, I.A., "The interrelationship of vitamin A
    and glucuronic acid in mucine metabolism", Science,
    85, 44-45 (1937).

5. Mori, S., "The changes in the para-occular glands which
    follow the administration of diets low in fat-soluble
    A; with notes on the effect of the same diets on the
    salivary glands and the mucosa of the larynx and
    trachea", John Hopkins Hospital Bulletin, 33,
    357-359 (1922).

6. Wolbach, B.S. and Howe, P.R., "Tissue changes following
    deprivation of fat soluble vitamin A", Journal of
    Experimental Medicine, 46, 699-706 (1927).

7. Goldblatt, H., and Benischek, M., "Vitamin A deficiency
    and metaplasia", Journal of Experimental Medicine,
    46, 699-706 (1927).

8. Sherman, H.C. and Mansell, H.E., "The quantitative
    determination of Vitamin A", Journal of American
    Chemical Society, 47, 1639-1646 (1925).

9. Green, H.N. and Mellanby, E., "Vitamin A as an anti-
    infective agent", British Medical Journal, 2, 691-696
    (1928).

10. Van Leersum, E.C., "Vitamin A deficiency and urolithia-
    sis",Journal of Biological Chemistry, 76, 137-142
    (1928).

11. McCarrison, H., "Stone formation in the bladder of rats
    on vitamin A deficient diets", Indian Journal of Medi-
    cal Research, 15, 801-806 (1927).

12. Mellanby, E.,"The Relation of diet to disease and health",
       British Medical Journal, 4, 677-680 (1930).

13. Kahn, R. H., "Effects of locally applied vitamin A
       and estrogen on the rat vagina", American Journal
       of Anatomy, 95, 309-328 (1954).

14. Lawrence, D. J., Bern, H. A., and Steadman, M. G.,
       "Vitamin A and keratinization", Annals of Otology,
       Rhinology and Laryngology, 69, 645-659 (1960).

15. Fell, H. B., and Mellanby, E., "Metaplasia produced
       in cultures of chick ectoderm by high vitamin A",
       Journal of Physiology, 119, 470-486 (1953).

16. Rothberg, S., "The cultivation of embryonic chicken
       skin in a chemically defined medium, and the response
       of the epidermis to excess of vitamin A", Journal of
       Investigative Dermatology, 49, 35-38 (1967).

17. Fell, H. B., and Mellanby, E., "The effect of hypervi-
       taminosis on embryonic limb bones cultivated in
       vitro", Journal of Physiology, 116, 320-346 (1952).

18. Parnell, J. P. and Sherman, B. S., in "Fundamentals
       of Keratinization", Butcher, E.O. and Sognnaes, R.F.,
       Ed., American Association for the Advancement of
       Science, Washington, D.C. (1962) Chapter 6

19. Saffiotti, U. et al, "Experimental cancer of the lung",
       Cancer, 20, 857-864 (1967).

20. DeLuca, L. and Wolf, G., in "Fat soluble vitamins",
       DeLuca, H.F., and Suttie, J.W., Ed., The University of
       Wisconsin, Press, Milwaukee, Wis.,(1970) Chapter 17

21. DeLuca, L. and Wolf, G., "Vitamin A and protein syn-
       thesis in mucous membrane", American Journal of
       Clinical Nutrition, 22, 1050-1062,(1969).

22. Prutkin, L., "Wound healing and vitamin A acid", Acta
       Dermato Venereologica, 52, 489-492 (1972).

23. DeLuca, L., Maestri, N., and Bonanni, F., "Maintenance
       of epithelial cell differentiation: the mode of
       action of vitamin A", Cancer, 30, 1326-1331 (1972).

24. Hutcher, N., Silverberg, S.G., and Lee, H.M., "The
       effect of vitamin A on the formation of steroid
       induced gastric ulcers", Surgical Forum, 22, 322-
       324 (1971).

25. Hunt, J. K., Garcia, J. A., Ehrlich, H. P., Dumphy, J. E., "The effect of vitamin A on reversing the inhibitory effect of cortisone on healing of open wounds in animals and man", Annals of Surgery, 170, 633-640 (1969).

26. Rai, K., and Courtemanche, A.D., "Vitamin A assay in burned patients", Journal of Trauma, 15, 419-424 (1975).

27. Dickson, J.A., in "Necrotizing enterocolitis in the Newborn Infant", Proceedings of the Sixty-eighth Ross Conference on Pediatric Research, Columbus, Ohio, Ross Laboratories, 1975, p. 9.

28. Roback, S. A., Foker, J., Frantz, I. F., Hunt, C. E., Engel, R. R. , Leonard, A. S., "Necrotizing entero-colitis", Archives of Surgery, 109, 314-319 (1974).

29. Santulli, T. V., Schullenger, J. N., Keird, W. C., Gongaware, R. D., Wigger, J., Barlow, B., Blanc, W.A., and Beaden, W.E., "Acute necrotizing entercolitis in infancy: a review of 64 cases", Pediatrics, 55, 376-387 (1975).

30. De, N.K.,"A fluorometric method for vitamin A assay", Indian Journal of Medicine Research, 43, 391-395 (1955).

31. Popper, H., "Histologic distribution of vitamin A in human organs under normal and under pathologic conditions", Archives of Pathology, 31, 766-802, (1953).

32. Fuyita, A., and Aoyama, M., "Colorimetric and fluoro-metric determination of vitamin A in fish liver oils", Journal of Biochemistry, 40, 157-168 (1953).

33. Kahan, J., "Fluorometric determination of vitamin A", Scandanavian Journal of Clinical and Laboratory Inves-tigations, 18, 679-690 (1966).

34. Carr, T.H., and Price, E.A., "Color reactions attri-buted to vitamin A", Biochemical Journal, 20, 497-501 (1926).

35. Bieri, J.G. and Schultz, M.O., "Comments on the micro-spectrophotometric determination of vitamin A and carotene in blood", Archives of Biochemistry, 34, 273-279 (1951).

36. Hansen, L.G., and Warwick, W.J., "A fluorometric micro-method for serum vitamin A", The American Journal of Clinical Pathology, 50, 525-529 (1968).

37. Thompson, J.N., Erdody, P., Brien, R., and Murray, T.K.,
    "Fluorometric determination of vitamin A in human blood
    and liver", Biochemical Medicine, 5, 67-89 (1971).

38. Thompson, J.N., Department of National Health and Welfare,
    Ottowa, Canida, private communication (1970).

39. Brandt, R., Olsen, M.J., and Cheronis, N.D.,"Apparent
    concentration quenching of morphine fluorescence",
    Science, 139, 1063-1064 (1963).

40. Orten, J.M. and Neuhaus, O.W., "Biochemistry", 8th ed.,
    C.V. Mosby Company, St.Louis, Mo., p. 767 (1970).

41. Goodman, D.S., "The transport protein for vitamin A in
    human plasma", Journal of Clinical Investigation, 47,
    2025-2037 (1968).

42. Jaarsveld, P.P., Edelhoch, H., Goodman, D.S. and Robbins,
    J., "The interaction of human plasma retinol binding
    protein and pre-albumin", Journal of Biological Chemis-
    try, 248, 4698-4705 (1973).

43. Thompson, J.N., Erdody, P., Maxwell, W.B., "Simultaneous
    fluorometric determinations of vitamin A and E in
    human serum and plasma", Biochemical Medicine, 8, 403-
    414 (1973).

44. Garry, P.J., Pollack, D., Owen, G.M., "Plasma vitamin A
    assay by fluorometry and use of a silicic acid column
    technique", Clinical Chemistry, 16, 766-772 (1970).

45. Brandt, R., Guyer, K., Blievernicht, R., Department of
    Biochemistry, Medical College of Virginia, unpublished
    data (1973).

46. Gal, J. and Parkinson, E.C., "Variations in the pattern
    of maternal serum vitamin A and carotenoids during
    human reproduction", E.C., International Journal for
    Vitamin and Nutritional Research, 42, 565-575 (1972).

47. Mueller, D., Department of Pediatrics, Medical College
    of Virginia, personal communication (1976).

48. Layne, E.,   in "Methods in Enzymology", Colowick, S.P.,
    and Kaplan, N. O., Ed., Academic Press, Inc., New York,
    Vol. III, 450-451 (1955).

49. Miller, G.M. and Cohen, W.D., in "Periodontal Therapy",
    5th ed., Goldman, H.M. Co. and Cohen, W.D., Ed., C.V.
    Mosby Company, St. Louis, Chapter 17 (1973).