Nutritional Elements
and
Clinical Biochemistry

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Edited by

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Proceedings of the Third Annual Meeting of the National Academy of Clinical
Biochemistry, held in New Orleans, Louisiana, July 13—14, 1979

Preparation of this volume has been an effort of the National
Academy of Clinical Biochemistry, derived in part from the program
of the third annual meeting, held in New Orleans, Louisiana,
July 13-14, 1979.

FOREWORD

The important role that the nutritional status exerts in
determining the course of life from birth to death in the human
being and especially its impact in disease states is only par-
tially appreciated at this time. Nutritional deficiencies are
usually considered to be major problems only in under-privileged
or developing populations, except for those occurring in specific
diseases. This attitude is incorrect as indicated by reports of
Bestrian et al (1974, 1976) and Merritt and Suskin (1979) and
others who found evidence of nutritional depletion in as much as
50% of the patients in varied groups of hospitalized patients in
the United States. Other studies, some of which are included in
this book, emphasized the existence of deficiencies of certain
specific nutrients.

Despite evidence of nutritional deficiencies occurring more
frequently than previously appreciated, there is no well established
protocol of laboratory studies that the clinical chemist or
scientist should provide to help the physician detect lack of
essential nutrients before extensive and possibly irreparable
damage has occurred to the individual patient.

Considerable research data are needed to determine the best
biologic material (i.e., erythrocytes, leucocytes, plasma, serum,
urine, cerebrospinal fluid, lymph) for analysis to determine
accurately at an early stage metabolic deficiencies due to a
specific nutritional element. Improved techniques for analysis of
key metabolites and nutrients are available in the research labora-
tory and many of them can be adapted to the clinical laboratory.

Need for accurate nutritional evaluation of the individual
patient has been greatly escalated in recent years. For example,
patients can be maintained for many years on hemodialysis programs,
for weeks or months by parenteral nutrition and on other programs
where adequacy of nutrition either due to inadequate absorption or
excessive metabolic waste may occur. Wisely selected laboratory
studies could be an aid to the physician in determining the type
and adequacy of the nutritional program of these difficult cases.

The scientific program of the third national meeting of the
National Academy of Clinical Biochemistry was devoted to a symposium
on Nutritional Elements in Clinical Biochemistry. Seven of these
speakers (D. Hill, J. Jones, H. Naito, P. Tocci, A. Dubin, R.
Shamberger and L. Lewis) expanded their symposium presentations,
and other authors kindly agreed to contribute greater breadth of
nutritional information.

It is hoped that the monograph, the first produced under NACB
auspices, will serve as a basic reference for current nutritional
knowledge in clinical biochemistry, and will help catalyze the
much needed research in nutritional assessment methodologies and
especially in the evaluation of alterations in nutritional status
secondary (or primary) to disease states and/or to their therapies.

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CONTENTS

CLINICAL CORRELATES IN NUTRITIONAL DISEASE

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INTRODUCTION

Many of the nutritional disorders of infants and children
such as rickets, scurvey, marasmus and kwashiorkor have been
recognized by their clinical and laboratory findings. Less well
appreciated are the abnormalities in growth due to major disturb-
ances in macronutrient intake, absorption and/or utilization that
result in failure-to-thrive at one end of the spectrum or obesity
at the other end. The origins of these growth abnormalities re-
lated to nutrition are frequently manifest in-utero or in infancy
and may be corrected by appropriate diagnosis and intervention.
The purpose of this article is to highlight some clinical examples
of failure-to-thrive and to contrast these with excessive growth
and obesity.

FETAL GROWTH

Factors that regulate fetal growth can be divided initially
into genetic and environmental influences. The genetic influence
can be examined by estimates of the effect on birth weight vari-
ance. Karn and Penrose (1951) have reported as much as 40% of the
variance due to the genetic effect with approximately one-half due
to the maternal genotype and one-half due to the fetal genotype.
The paternal influences are mediated through the fetal autosomal
genes and sex of the infant. Another approach that provides an
index of genetic influence is to examine populations of infants
with chromosome anomalies. Both autosomal anomalies and some sex
chromosome anomalies result in a lower average birth weight than
in controls (Table I). Polani (1974) emphasizes the strong in-
fluence of the X chromosome and the mild influence of the Y

Table I. Mean Birth Weights for Chromosomal Disorders

Triploidy	2.1 kg	(36 weeks gestation)
Trisomy 18 syndrome	2.26 kg	
Trisomy 13 syndrome	2.6 kg	
5p – syndrome	2.66 kg	
18q – syndrome	2.86 kg	
19p – syndrome	2.87 kg	
XO Turner syndrome	2.9 kg	
Down's syndrome	3.06 kg	
XXY syndrome	3.15 kg	
XYY syndrome	3.36 kg	

(Data from Polani, 1974)

chromosome on fetal growth. The absence of an X chromosome may reduce the birth weight by as much as 500 grams or, if an extra X chromosome is present, the birth weight may be increased by up to 300 grams.

The remaining 60% of the variance in birth weight can be accounted for by the environment in which the fetus grows. Further subdivided; maternal size, prepregnant weight, weight gain during pregnancy, age, parity, and general health may account for 20-30% of this variance (Elliot and Knight, 1974). We are left with approximately 30% of the variance in birth weight due to unknown factors in any one pregnancy. Some of the external influences, such as smoking, alcohol, other drugs, infection, and severe under-nutrition, may be recognized in a specific instance as the major etiologic agent producing significant fetal growth retardation.

Vascular Factors

If one attempts to classify the causes of fetal growth re-tardation, it is evident that the majority of known causes are related to vascular disorders in the mother (Table II) (Elliot and Knight, 1974). The emphasis on etiology has not promoted extensive investigation of the mechanism involved in reducing fetal growth. One obvious need is to examine carefully the vascular supply to the fetus since most of the nutrient transfer is flow related. The utero-placental blood flow can be influenced by a number of factors, and reductions in maternal systemic perfusion produce a propor-tional disease in uterine blood flow. During pregnancy, the uterine vascular bed seems maximally dilated and is relatively unresponsive to further stimuli such as reduced perfusion pressure, hypoxia, or hyperoxia. In addition, estrogens have little effect

Table II. Suggested Percentage Distribution of Causes
           of Small-for-Dates Babies in the UK

CAUSE

Normal variation	10
Chromosomal and other congenital anomalies	10
Infections (maternal and fetal)	5
	(perhaps < 5?)
Poor uterus	1
Placenta and cord	2
Vascular disease in the mother	35
Drugs, medicaments and smoking	5
Other	32

(From Elliot and Knight, 1974)

late in pregnancy on uterine vasculature. There is little re-
sponse to beta-adrenergic stimulation, but stimulation of the
sympathetic nervous system results in vasoconstriction and reduced
uterine flow (Burd, 1976).

The presumed cause of reduced fetal growth in maternal vascu-
lar disease is compromised uteroplacental perfusion and a decrease
in trophoblastic surface area. Since the placenta acts as an
organ of nutrient exchange, and flow and permeability influence
the placental clearance of molecules across the placenta, more
attention needs to be directed toward the assessment of nutrient
transfer to the fetus. Except under unusual circumstances, sub-
strate availability in the maternal circulation could not be
viewed as the rate limiting process in fetal growth. More likely,
a reduction in uterine blood flow and therefore placental transfer
is operant in the majority of instances. Placental clearance can
be measured in experimental animals by calculating (see Table III)
umbilical blood flow and determining the arterial-venous concen-
tration difference of the particular substrate (Burd, 1976).

However, good estimates of placental clearance or capacity
are difficult to obtain in humans. In some laboratories, the clear-
ance of dehydroepiandrosterone sulphate (DHEAS) is used as an assess-
ment of placental function (Worly, 1975) and has been helpful in
identifying those patients with significant reduction in placental
function prior to overt clinical signs of pregnancy hypertension.
There are a number of limitations to this test and clearly there
is a need for development of a good, safe, reliable, non-invasive
technique for assessing placental function.

Table III. Placental Clearance for a Compound that is
            Transferred from Fetus to Mother

$$C_{Placental \atop Clearance} = \frac{F_{Umbilical \atop Blood\ Flow} \times (A_{1\ Umbilical \atop Arterial\ Conc.} - V_{1\ Umbilical \atop Venous\ Conc.})}{A_{1\ Umbilical \atop Arterial\ Conc.} - A_{2\ Uterine \atop Arterial\ Conc.}}$$

(From Burd, L.I., 1976)

Hormonal Factors

In addition to an adequate blood supply, the fetus must pro-
duce a number of polypeptide hormones to grow adequately. Most of
the attention has been directed toward fetal thyroid hormone and
growth hormone or somatomedin. These hormones have little in-
fluence on overall somatic growth in utero, while they are impor-
tant for maturation and possibly altering substrate supply. More
recently insulin has gained prominence as a major regulator of
fetal growth, particularly during the latter part of the third
trimester (Hill, 1976, 1978). Both clinical and experimental data
support a role for insulin during fetal life as essential for
fetal growth (Table IV). The classic example of the insulin effect
on fetal growth is the infant-of-the-diabetic-mother (IDM).

Table IV. Insulin Effects on Fetal Growth

Hyperinsulinemia or Increased Sensitivity	Hypoinsulinemia or Decreased Sensitivity
Infant of the Diabetic Mother	Transient Diabetes Mellitus
Insulinoma and Nesidioblastosis	Pancreatic Agenesis
Beckwith-Wiedemann Syndrome	End Organ Defect (Receptor)
Infant of the Gestational Diabetic	Intrauterine Growth Retardation

In this instance, poor control of the diabetes in the mother re-
sults in large oscillations in blood glucose that are directly
transferred to the fetus. The fetal beta cell responds to the

glucose stimulus by hypertrophy and hyperplasia and increased
insulin production. As the insulin output increases, there is
overgrowth of almost all fetal tissues with the most striking
changes occurring in the adipose tissue, liver, and muscle
(Fig. 1). The infant becomes dependent on the relatively high
blood gluocse and when delivered, has a precipitious fall in glu-
cose and frequently has symptomatic hypoglycemia. It is important
to recognize that the fetal hypersomatism and the induced meta-
bolic changes can be abolished by meticulous control of the mater-
nal diabetes. Prognosis for normal growth in these infants is
good and a slowing in growth velocity occurs during the first two
years of postnatal life. These infants are good examples of how
alterations in substrate supply (glucose) can induce hormonal
changes and altered growth in the fetus, as well as indicating how
excessive fetal growth can be transient and return to normal. In
severe maternal diabetes with vascular disease (White Class F),
the reduction in uterine blood flow and placental transfer of
substrate is such that the majority of infants from these patients
are growth retarded.
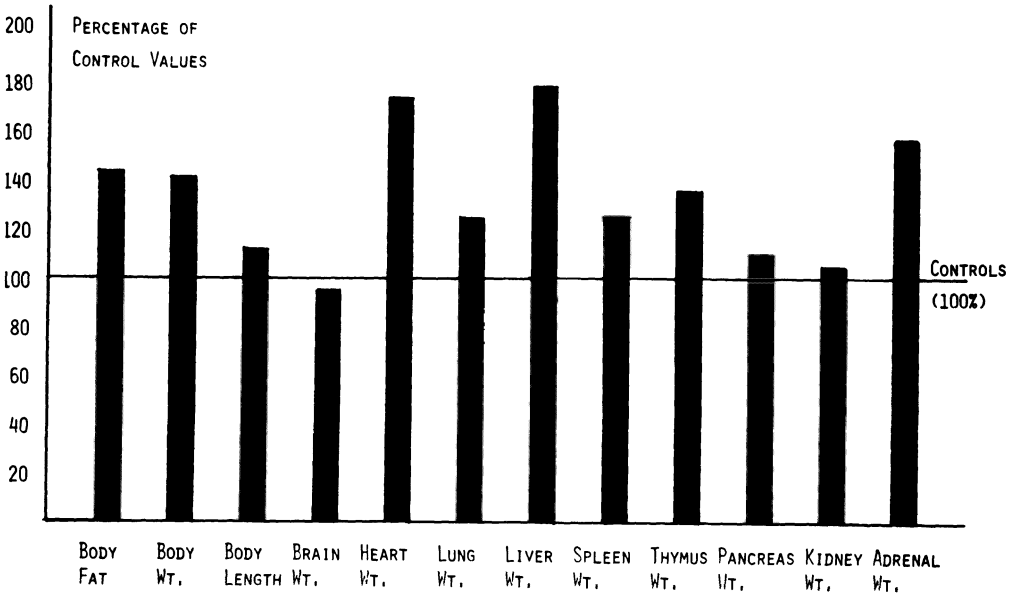
Fig. 1. The body weight and organ weight are shown for some
         infants of diabetic mothers compared with controls.
         (Re-drawn from Osler and Petersen, 1960 and Naeye,
         (1965).

Other examples of fetal hypersomatism are listed in Table IV and are relatively rare, but frequently have a very similar clinical appearance to the IDM and have excess de novo insulin production. In these instances, neonatal hypoglycemia usually persists until the pancreatic overproduction of insulin is ablated through drugs or surgery. The prognosis for normal growth is good.

Contrasted with the IDM and other forms of insulin overproduction is the group of patients who have insufficient or total absence of insulin and intrauterine growth retardation. The classic cases of total pancreatic agenesis (Sherwood et al., 1974) offer the opportunity to examine how fetal growth is arrested in the absence of fetal insulin production. These infants obtain a size of approximately 30 weeks gestation and do not have adequate development of muscle, adipose tissue, and liver. They behave as diabetics once they are born and can no longer depend on maternal glucose homeostasis for control. In addition, a large number of growth retarded infants with maturational lag in pancreatic islet function have been described. These infants develop diabetes mellitus and may die if not treated appropriately. With good care and insulin therapy, catch-up growth is dramatic and most infants recover their own pancreatic function.

The above cases are not known to have any particular problem in vascular supply or substrate transfer, but they clearly have an inability to utilize substrate for developing muscle, adipose tissue, and glycogen. Thus, the interaction between substrate and hormones for optimal fetal growth.

Nutrition

The growth of the fetus obviously depends on the availability of an adequate nutrient supply. Until recently, glucose was considered the major metabolic fuel of the fetus (Simmons, 1976). Studies in sheep where catheters have been implanted in fetal arteries and veins and maintained during pregnancy (Simmons et al., 1974), have indicated that glucose may account for only 45% of oxygen consumption and the remainder comes from amino acids and from lactate (Table V). If these findings can be extrapolated to humans, there is need, therefore, of a continuing supply of glucose, amino acid, and lactate.

Considerable controversy exists over the concept of limiting or reducing fetal growth by reducing maternal intake of calories and protein and other nutrients. The vast majority of evidence that some influence on fetal growth can be produced by restricting maternal intake comes from data on rats (Winick, 1972). In humans and non-human primates, it is difficult to demonstrate a direct relationship between maternal nutrient intake and fetal growth (Cheek, 1975). In North America, where undernutrition is usually

Table V. Contributions of Various Substrates to Fetal
          Oxidative Catabolism

Glucose	0.45
Amino acids	0.25
Glycerol	0.01
Fructose	0.01
Free fatty acids (C > 5)	0.01
Lactate	0.25
	1.00

(From Simmons, 1976)

coincident with poor socioeconomic conditions, it is nearly im-
possible to separate nutrient intake as a single variable and
demonstrate a rate-limiting effect on fetal growth. Similarly,
if one examines the number of growth retarded infants associated
with significant undernutrition in the mother, the total number
is fewer than 1%. There appears to be a margin of safety for the
fetus as exemplified in some cases of severe undernutrition with
normal or slightly reduced birth weights (Stein and Susser, 1975).
In studies on the effects of dietary supplementation, there is a
modest (40-60 gram) increase in the average birth weight and only
in those women who were thin and undernourished at the beginning
of the study (Stein et al., 1978). The Dutch famine study supports
the notion that a sparse but well balanced diet had little or no
relation to fetal growth until there was severe food deprivation.

These findings do not negate the possibility that a fetus may
be marginally nourished or malnourished in-utero in the presence
of a normally or optimally nourished mother. As emphasized in the
section on vascular or hormonal factors, the basic nutrient supply
to the fetus may be compromised by poor uterine and umbilical flow
and by alterations in assimilation of the substrate.

Classification of Altered Fetal Growth

The first good documentation of the undernourished full-term
infant appeared in an article by McBurney (1947). Since that time
there have been numerous growth curves established for the fetus
related to gestational age. The most familiar of these is that
produced from the Denver population (Fig. 2) (Battalgia and
Lubchenco, 1967). While this graph is frequently used, others from
Baltimore (Gruenwald, 1964) and Montreal (Usher and McLean, 1969)
as well as local standards should be used since there are definite
regional and ethnic differences in birth weights. In any of these

GRAMS
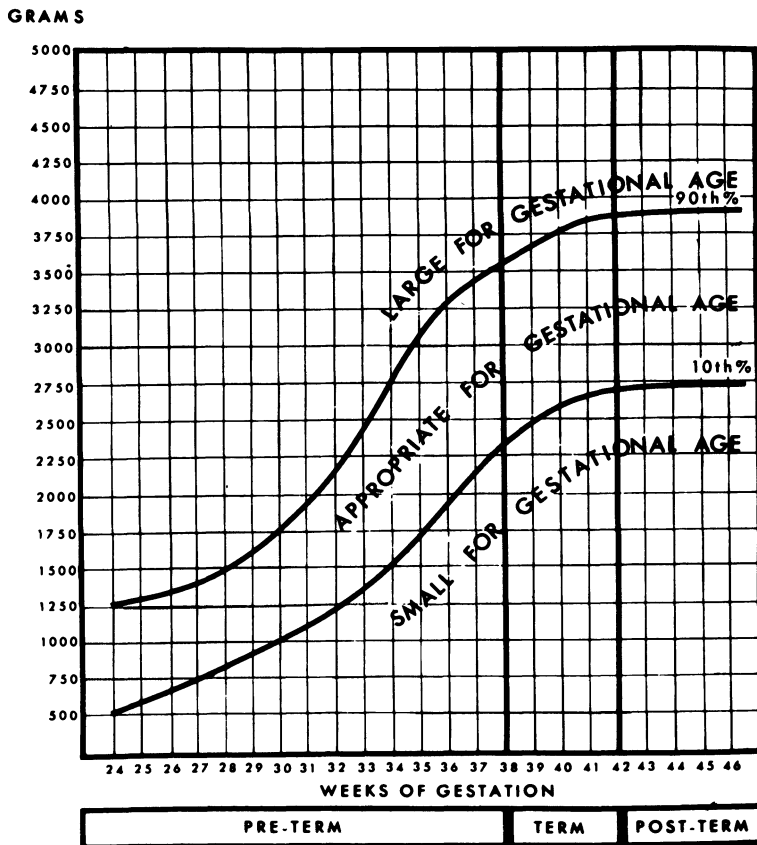
Fig. 2. The classification of newborns by birth weight and
         gestational age commonly used in newborn nurseries.
         (Battaglia and Lubchenco, 1967).

growth grids, the important point is to properly determine the
gestational age of the newborn and to determine if the infant is
preterm, term, or post-term and then to determine if the infant is
large-for-gestational-age (LGA), appropriate-for-gestational-age
(AGA), or small-for-gestational-age (SGA). This initial

classification should be done for weight, length, and head circum-
ference. Once it has been determined that the infant is SGA, then
a search for the etiology can proceed. In approximately 30% of
patients, the pattern of growth established prenatally will persist
postnatally and the infant and child continue to exhibit low pro-
file growth at or below the 10th percentile. Usually, no specific
cause can be found for their poor growth. The remaining infants
have variable degrees of catch-up growth often accompanied by an
excessive nutrient intake for body size or age. These infants are
no longer under constraint and consume nutrients to satisfy the
increased demand for growth. Many of these infants cross percen-
tile lines and return to their individual genetic pattern or
channel for growth.

     It is evident, therefore, that the SGA infants have hetero-
genous etiologies that produce growth failure in-utero and the
eventual prognosis for postnatal growth is very much determined by
the specific etiologic agent. In those instances where cell re-
plication is inhibited or arrested for a significantly long period,
such as in rubella or cytomegalic inclusion disease, the growth
retardation is usually permanent. Conversely, if the constraint
to growth is due to a reduced blood supply late in gestation,
there is usually rapid and complete postnatal catch-up growth. If
more effort is expended on properly classifying newborn infants
according to body proportions and birth weight for gestational age,
then prognostication on ultimate growth and performance of the
child will become easier.

POSTNATAL GROWTH

     One of the most frequent reasons for a child to be referred
for assessment is because of an abnormality in his or her post-
natal growth. As mentioned in the first few paragraphs, these
growth abnormalities may be at either end of the spectrum, i.e.,
failure-to-thrive or obesity. Usually, a detailed history in-
cluding the obstetric and neonatal history will provide some
evidence of growth abnormality prior to birth. In those infants
who fail-to-thrive after a normal prenatal and birth history,
there is frequently a history of disturbed nutritional intake or
assimilation (Table VI). When these infants are fed in a con-
trolled environment, they will often experience dramatic catch-up
growth. Seldom is there an organic cause for their failure-to-
thrive, but if, in fact, they have a major system disease, the
prognosis for normal growth is closely related to the severity of
the disease. In addition, disorders such as renal failure or
severe asthma are often treated with growth retarding drugs such
as corticosteroids. When the corticosteroids are reduced or dis-
continued, there is frequently some catch-up growth. There is
little evidence that nutrition, per se, is the rate-limiting

Table VI. Causes of Failure to Thrive in a Hospital
           Population

	No.	Percent
Suboptimal nutrition	174	87
Other cases:		
Cystic fibrosis	6	3
Hypothyroid	5	2.5
Subdural hematoma	5	2.5
Glycogen storage disease	1	0.5
Celiac syndrome	1	0.5
Methylmalonic acidemia	1	0.5
Maple-syrup urine disease	1	0.5
Unknown	6	3
TOTAL	200	100

factor although some improved growth can be witnessed following
caloric supplementation in chronic renal disease. True endocrine
abnormalities such as hypothyroidism or hypopituitarism are rare
causes of growth failure in children. Usually, a good history
plus the appearance of the child and his or her bone age will
suggest one of these endocrinopathies. The nutrient intake in
these children is commensurate with the rate of growth of the
child. Abnormalities of insulin such as juvenile diabetes
mellitus can produce growth retardation if poorly controlled.

Excessive growth during the first few years postnatally is
usually manifest by an abnormal pattern of weight gain due to
adipose tissue. This has become a major concern in pediatrics
since there is evidence that a relatively high percent of obese
infants become obese children and obese adults (Heald and Hollander,
1965). The factors causing this increase in the incidence of
obesity in infants and children are not clear. Inherited factors
play an important role, but clear-cut inheritance patterns have not
been established. Some studies claim that bottle feeding plus the
early introduction of solid foods have a major role in the pro-
duction of obesity in infants. Work completed in our clinic
(Dubois et al., 1979) does not support this contention and suggests
that individual differences in energy storage and utilization must
be important since obese infants had a lower caloric intake per
kilogram than the non-obese infants. The same finding has been
reported in older obese children and adults.

It would appear that individual disturbances of hypothalamic regulation may result in morbid obesity or conversely, in anorexia and cachexia. There are both human and experimental data illustrating how damage to the ventromedial hypothalamus can produce hyperphagia and obesity (Martin et al., 1974). These conditions are usually associated with excessive insulin secretion that precedes the hyperphagia and obesity (Martin et al., 1974). A peptide isolated from the ventrolateral hypothalamus of rats and monkey has a very specific stimulatory effect on pancreatic insulin production (Hill et al., 1977). This peptide has not yet been characterized, but in crude form is present in the plasma of obese animals. It may mediate the lipogenic effect through insulin stimulation.

While there is evidence of central hypothalamic disturbance in some children, these are the rarer causes of obesity. It must be concluded that numerous factors enter into the equation resulting in obesity, but each child who becomes obese has exceeded the energy requirement for normal basal metabolism and growth during a significant period of development.

SUMMARY

Abnormalities in both prenatal and postnatal growth have been highlighted. Disturbances in the intake, assimilation, or metabolism of macronutrients appear to be the common denominator in a number of these disorders. The challenge to the biochemist and clinical investigator is in at least four areas:

1) Development of non-invasive, safe techniques for the assessment of placental transfer and function.

2) Further elucidation of the role of insulin in both prenatal and postnatal growth.

3) Assessment of the rate of intrinsic cell growth and alterations with nutritional manipulations.

4) Clearer definition of the individual metabolic differences in the obese versus the non-obese child.

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CLINICAL SIGNIFICANCE OF RIBOFLAVIN DEFICIENCY

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INTRODUCTION

The clinical consequences of riboflavin deficiency in humans
were first described by Sebrell and Butler (1938). On a diet which
furnished only 0.5 mg of riboflavin daily, 10 of 18 adult women
developed oral lesions that disappeared following riboflavin
supplementation. Now, over 40 years later, riboflavin deficiency
is recognized as a global health problem that can occur in persons
of any race, sex or age. Much new knowledge about riboflavin has
been obtained during the last 10 years due to major improvements
in methods for measuring riboflavin status and a concomitant re-
newed interest in nutrition. This paper will review the clinical
significance of riboflavin deficiency with a special emphasis
being given to recently acquired information.

STRUCTURES AND CHEMICAL-PHYSICAL PROPERTIES

The history of riboflavin discovery, isolation and characteri-
zation has been reviewed in detail (Gyorgy, 1954; Wagner-Jauregg,
1954; Gyorgy, 1967). Karren et al. (1935) and Kuhn et al. (1935)
independently synthesized riboflavin and proved its structure to
be 7,8-dimethyl-10-(1'-D-ribityl) isoalloxazine (Figure 1). The
name, riboflavin, thus reflects the presence of D-ribitol and
flavin. Riboflavin is commonly referred to as vitamin B_2 and was
earlier called vitamin G in the United States. Other previous
names for riboflavin include lactoflavin, ovoflavin, uroflavin and

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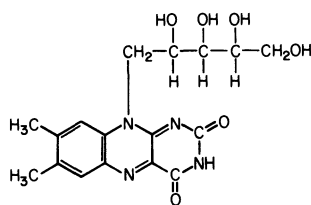
RIBOFLAVIN

Figure 1.
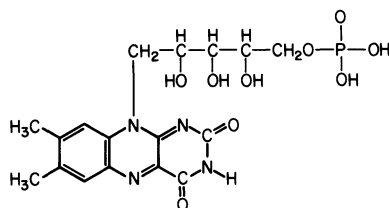
RIBOFLAVIN 5-PHOSPHATE (FMN)
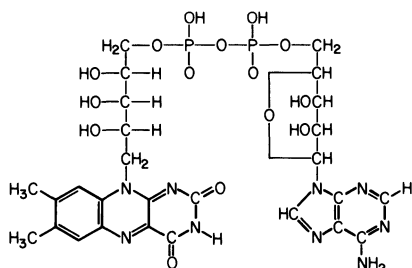
FLAVIN ADENINE DINUCLEOTIDE (FAD)

Figure 2.

hepatoflavin which reflect the source of the vitamin. The princi-
pal forms of riboflavin found in nature are riboflavin 5'-phosphate
(FMN) and flavin adenine dinucleotide (FAD) (Figure 2).

Riboflavin, FMN and FAD have molecular weights of 376, 456 and 786, respectively. Flavins have a characteristic yellow color resulting from strong absorption in the violet and blue regions. In crystalline form flavins are stable to heating up to 100-120°. Riboflavin is odorless but has a bitter taste. It is poorly soluble in water (0.03 - 0.15 g/100 ml), perhaps accounting for its low toxicity. A saturated solution has a pH of approximately 6. FMN and its sodium salt are highly soluble and FAD is very hygroscopic and is freely soluble. All three compounds have multiple absorption peaks and exhibit intense greenish-yellow fluoresence (riboflavin and FMN) and orange fluoresence (FAD).

In aqueous solution flavins are relatively heat stable in the absence of light. Flavins present in food are unaffected by baking in an oven below 500°F (Williams and Cheldelin, 1942; Mayfield and Hedrick, 1949) or short-term sterilization by autoclaving (Wagner-jauregg, 1972). In contrast, the riboflavin content of milk is reduced by more than 50% within two hours of exposure to sunlight (Ziegler, 1944). Flavins are relatively stable against common oxidizing agents. Generally, acids have no destructive influence on the isoalloxazine nucleus but the ester bond of the nucleotides is readily hydrolyzed. FAD can be fully hydrolyzed to FMN by allowing it to stand overnight at 38°C in 10% trichloroacetic acid although no destruction occurs at 0°C for 30 minutes. All flavins undergo decomposition in the presence of alkalies; if irradiated by light, lumiflavin and lumichrome appear as photolytic products. Several excellent reviews of the physical and chemical properties of flavins are available (Yagi, 1962; Wagner-Jauregg, 1972; Merrill and McCormick, 1980).

METABOLISM AND FUNCTIONS

Riboflavin is consumed primarily as FMN- and FAD-protein complexes (McCormick, 1975). Prior to absorption the flavins are released by gastric acidification and proteolysis. Flavins are absorbed from the proximal region of the small intestine by a specialized site-specific and saturable transport process (Campbell and Morrison, 1963; Levy and Jusko, 1966; Jusko and Levy, 1967). Before flavins can be taken up by the intestine, it is necessary that they be dephosphorylated in the intestinal lumen. Prior to their release in the blood, the flavins are rephosphorylated in the mucosal cells.

Man is fairly efficient in absorbing riboflavin but concomitant ingestion with meals provides maximal absorption. The effect of food upon enhancing absorption may be due to the release of bile salts, agents that appear to facilitate the absorption of flavins (Mayersohn et al., 1969). When low levels of flavins are fed to normal persons, the amounts recovered in the urine are proportional to the amounts ingested. There is, however, an upper limit in

intestinal absorption of riboflavin of about 25 mg (Jusko and Levy, 1967). When 30 mg of FMN or more are given orally, only about 50% is absorbed. Neither the mechanism underlying this latter obser- vation nor its possible regulatory role are known.

Flavins are present in all biological fluids and tissues. After entering the circulation a large fraction of riboflavin and FMN is bound to albumin in a rather weak association (Jusko and Levy, 1969). Numerous other plasma protein fractions also bind riboflavin. Very recent work has described the purification of riboflavin-binding proteins from bovine plasma that migrate electro- phoretically with the globulin fraction (Merrill et al., 1979). Preliminary work indicates the presence of comparable binding proteins in human plasma (Merrill and McCormick, 1980).

The flavins are quickly transported to the various tissues with the majority being taken up by the liver and kidneys leaving very little in the blood (Baker et al., 1966; Yagi et al., 1966; Fazekas and Sandor, 1973). Most tissues contain 1 μg flavin/g tissue or less (Koziol, 1971). In the tissues riboflavin is con- verted by flavokinase to FMN (McCormick, 1961; Merrill et al., 1978) which is subsequently converted to FAD by a pyrophosphorylase (McCormick, 1964). Some studies have indicated that thyroid hor- mones are involved in maintaining flavin coenzyme levels (Rivlin et al., 1968). FAD is the predominant flavin in tissues (Cerletti and Ipata, 1960; Yagi, 1962). The interconversions of riboflavin, FMN and FAD have been extensively reviewed by McCormick (1975).

Renal excretion of riboflavin involves glomerular filtration, tubular secretion and reabsorption (Morrison and Campbell, 1960a). The amount excreted depends not only on the amount ingested but also on the levels present in the body. When radioactive ribo- flavin is injected, about 81% of the radioactivity is retained after 24 hours (Yang and McCormick, 1967). Free riboflavin is the only flavin excreted from the body in significant amounts. The main excretory route is the urine with very minor amounts being excreted in the bile, feces, sweat and breast milk. There are trace levels of flavin catabolites present in the urine (West and Owen, 1969) which probably arise from intestinal flora (Yang and McCormick, 1967).

FMN and FAD form the prosthetic groups of several different enzyme systems which are collectively known as flavoproteins. They participate in numerous reactions (Horwitt and Witting, 1972; Merrill and McCormick, 1980). The oxidation of D- and L-amino acids by the corresponding amino acid oxidases requires FAD and FMN, respectively. In carbohydrate metabolism FMN acts in concert with pyridine nucleotides to oxidize glucose-6-phosphate and produce energy. Xanthine oxidase and aldehyde dehydrogenase are additional examples of enzymes involved in aerobic oxidation that require

flavoproteins. FMN and FAD are also cofactors in several mito-
chondrial anaerobic dehydrogenation reactions. Examples of the
latter include the transfer of hydrogen from NADH to the cyto-
chromes, succinic dehydrogenase in the tri-carboxylic acid cycle
and the β-oxidation of fatty acids via acyl-CoA dehydrogenases.

Most of the flavins found in blood are present in the erythro-
cytes with FAD being the predominant form (Burch et al., 1948).
Since erythrocytes lack mitochondria, there are only a limited
number of flavin enzymes present in these cells. One important
role of FAD is as a coenzyme of glutathione reductase (EGR) which
ensures proper maintenance of reduced glutathione (GSH) levels.
The other FAD flavoprotein in red cells is methemoglobin reductase.

In pregnancy the FAD present in the maternal circulation does
not cross the placenta but is degraded to free riboflavin in the
placenta for secretion into the fetal blood (Lust et al., 1954).
Riboflavin is also present in amniotic fluid (Clarke, 1973a).
Umbilical cord blood levels at delivery are related to maternal
blood levels but are always higher in the neonates - even in face
of maternal hypovitaminosis (Kaminetzky et al., 1973, 1974).
Several workers have confirmed the finding that neonates invariably
have better riboflavin status than their mothers (Bamji, 1976;
Baker et al., 1977; Clarke, 1977b). This disproportionate distri-
bution of flavins between the mother and her fetus may be due to a
carrier-mediated transport mechanism (Nutr. Rev., 1979). Although
not yet demonstrated in the human, pregnancy-specific riboflavin-
binding proteins have been shown to occur in chicks (Murthy and
Adiga, 1977a,b,c, 1978), rats (Adiga and Muniyappa, 1978) and cows
(Merrill et al., 1979). Furthermore, estrogen appears to induce
their synthesis in liver and modulate their levels in plasma.

Accompanying the weight loss that normally occurs in a neonate
during the first 3 days of life after birth is a high urinary ex-
cretion of riboflavin (Hamil et al., 1947). These large amounts
excreted early in life may partly reflect the intrauterine storage
as well as the negative nitrogen balance that usually occurs in the
first few days of life. Urinary excretion of riboflavin decreases
as the neonate undergoes transition to the extrauterine environment
and milk intake is increased.

REQUIREMENTS

Man must have a constant supply of riboflavin for the optimal
maintenance of the many metabolic processes involving the flavin
coenzymes (Tillotson and Baker, 1972). This need is met in adults
when dietary riboflavin intake is sufficient to keep the body
tissues near-saturated by replacing riboflavin lost from the body.
During physiological stress periods of growth, pregnancy and lacta-
tion, riboflavin requirements are increased. Recommendations for

the dietary riboflavin intake of healthy normal people are set at
levels which will not only prevent the deficiency syndrome of
ariboflavinosis but will ensure maximal functional capacity and
meet any needs due to physiological stress.

The bulk of the data used to establish riboflavin requirements
has been derived from balance studies. An important consideration
in this regard is that meaningful measurements of urinary ribo-
flavin excretion cannot be obtained in individuals with negative
nitrogen balance (Oldham et al., 1947; Pollack and Bookman, 1951;
Bro-Rasmussen, 1958; Smith et al., 1959). Inadequate protein
intake and disease are invariably accompanied by negative nitrogen
balance. This is a major reason why requirements are known only
in normal, healthy individuals. Other factors which can result in
altered nitrogen metabolism are discussed in the section on Assess-
ment (Biochemical, Assay Methods) and some of the diseases known to
decrease the levels of riboflavin are summarized in the section on
Occurrence of Riboflavin Deficiency. There is little evidence,
however, that disease per se causes an increased need for water-
soluble vitamins (Coon, 1965).

There have been several suggestions for the best way to ex-
press riboflavin requirements. Arguments for estimating riboflavin
needs in terms of caloric consumption (Bro-Rasmussen, 1958) and
protein requirements (Horwitt, 1966) have been critically reviewed.
The World Health Organization (1965, 1967 and 1974) has chosen to
relate riboflavin needs to caloric intake at a level of 0.55 mg/
1000 kcal and makes no additional adjustments for riboflavin re-
quirements during physiological stresses. This value is similar to
the riboflavin intake which maximizes the level of EGR (Beutler,
1969). The Food and Nutrition Board of the National Acacemy of
Sciences (RDA, 1980) expresses riboflavin requirement in terms of
metabolic body sizes, e.g., the adult requirement is 0.07 mg per
day per Kg $^{0.75}$ If related to calorie intake, the RDA are higher
than the WHO recommendations.

The early studies of Horwitt et al., (1949, 1950) suggested
that the riboflavin requirement of the resting adult is 1.1 to 1.6
mg per day. Intakes below 0.5 mg per day are insufficient to support
normal tissue repair and will result in ariboflavinosis (Bro-
Rasmussen, 1958). The needs of infants and young children were
initially evaluated to be approximately 0.4 mg per day (Oldham
et al., 1944; Snyderman et al., 1949). These values for human
riboflavin requirements have stood the test of time well (See
Table I).

Some studies suggest no increased requirement for riboflavin
with the progress of pregnancy (Oldham et al., 1950) but other
reports of decreased urinary excretion (Dubrausky and Blazso, 1943;
Brzezinski et al., 1952), reduced whole blood levels (Clarke, 1971),

Table I. Daily Riboflavin Requirement

Group	Age (Year)	Weight (Kg)	Energy (Kcal)	Recommended Dietary Allowances (1980)* mg/daily
Infants	0-0.5	6	Kg. X 115	0.4
	0.5-1.0	9	Kg. X 105	0.6
Children	1-3	13	1300	0.8
	4-6	20	1700	1.0
	7-10	28	2400	1.4
Males	11-14	45	2700	1.6
	15-18	66	2800	1.7
	19-22	70	2900	1.7
	23-50	70	2700	1.6
	51-75	70	2400	1.4
	76+	70	2050	1.4
Females	11-14	46	2200	1.3
	15-18	55	2100	1.3
	19-22	55	2100	1.3
	23-50	55	2000	1.2
	51	55	1800	1.2
	76+	55	1600	1.2
Pregnancy			+ 300	+ 0.3
Lactation			+ 500	+ 0.5

*These allowances are intended to provide for individual
variations among most normal persons as they live in the
United States under usual environmental stresses.

enzyme tests indicating riboflavin deficiency (Heller et al., 1974)
and an increased incidence of deficiency syndromes (Brzezinski
et al., 1952) clearly indicate an increased need. Lactation also
increases riboflavin requirements (Roderuck et al., 1946). The
increase in requirement for B-vitamins during pregnancy,

particularly in the final trimester, may be due partly to seques-
tration of vitamins by the fetus and placenta, and partly to the
metabolic effects of high circulating levels of female sex hor-
mones (Bamji, 1976).

The best food sources of riboflavin are milk, liver, eggs,
meat and some of the green leafy vegetables (Horwitt, 1972; Foy
and Mbaya, 1977). One quart of milk contains about 2.0 mg of
riboflavin which is equivalent to approximately two pounds of lean
meat. Yeast is the richest natural source but does not contribute
significantly to dietary intake. Unenriched grains and legumes
are poor sources but supply important amounts to many regimens due
to large consumption of these foods. Grains have become valuable
riboflavin sources in the United States because of bread enrich-
ment programs. The riboflavin intake of many diets is related to
the amount of ingested animal protein. Riboflavin consumption in
the United States increased from 1.9 in 1935 to 2.3 mg/person/day
in 1959, mostly due to greater consumption of beef (Foy and Mbaya,
1977). Today's current market prices for meat have undoubtedly
reduced riboflavin intake from this source. Non-food sources of
riboflavin include vitamin synthesis by intestinal flora (Najjar
et al., 1944) but the amounts produced are small and may not be
taken up by the body.

ASSESSMENT OF RIBOFLAVIN STATUS

The methods used for assessing nutritional riboflavin status
in man are comparable to those used for other vitamins. There are
several very good summaries concerning nutritional assessment
techniques (Goldsmith, 1959, 1964, 1975; Jelliffee, 1966; Ten
State Nutrition Survey, 1968-1970; Christakis, 1973). A diagnosis
of riboflavin deficiency is usually made on the basis of a pro-
longed history of poor riboflavin intake which may or may not be
accompanied by clinical signs and symptoms of ariboflavinosis.
Final confirmation is accomplished by biochemical evaluation. In
those instances where laboratory tests are not available, therapeu-
tic diagnosis might be considered. It is essential to know nutri-
tional requirements, be familiar with nutrient functions, be
cognizant of the potential influence of disease on nutrient require-
ments and utilization and to evaluate possible biochemical dis-
turbances in the body.

In population groups with very uniform food patterns, which is
often the case in developing countries, relatively simple methods
yield good results (Brubacher, 1974). In this situation the aim
is to decide if there is a public health problem. Methods can thus
be used which are applicable to screening large population groups.
In highly industrialized countries, however, more sophisticated
techniques are needed which can be applied to individuals in a
clinical setting. It is unfortunate but there is generally a poor

correlation between dietary, clinical and biochemical evaluation
of riboflavin nutriture (Axelrod et al., 1941; Brocklehurst et al.,
1968; Thurnham et al., 1970; Buzina et al., 1971, 1973; Sharada
and Bamji, 1972; Schorah and Messenger, 1975; Kaufmann and
Guggenheim, 1977). Only when the riboflavin deficiency is pro-
longed and severe is there a very good correlation between these
variables (Bamji, 1969; Beutler, 19 9; Tillotson and Baker, 1972;
Vir and Love, 1977).

Dietary

     An estimate of riboflavin status can be made by determining
dietary intake of the vitamin and comparing this with an indivi-
dual's needs as discussed in the previous section. An indication
of adequate riboflavin intake does not insure that there are
adequate body stores but does imply minimal risk for developing
ariboflavinosis. Dietary methods are laborious and expensive.
They are particularly useful in nutrition surveys for screening
large numbers of people and for monitoring hospitalized patients.

     There are several methods by which to evaluate dietary intake.
The most commonly utilized approach is the diet record. The sub-
ject records types and amounts of food consumed over some specified
period of time. Alternately, this same information can be obtained
by interviews. More exacting information results when home visits
are included in the evaluation. Each of these methods requires
the use of food composition tables which often contain inadequate
or questionable values. The most precise approach involves the
actual determination of the amount of riboflavin present in 24-hour
food composites by the same basic assay methods in the Biochemical
Section. Availability of this latter method is, however, extremely
limited.

Clinical

     Few clinical signs have pathognomic significance. Unlike some
of the other nutrient deficiencies, ariboflavinosis is not a life
threatening situation. Only the general aspects of the clinical
evaluation of riboflavin status will now be discussed. Specific
clinical manifestations of ariboflavinosis are described in the
section on Effects of Riboflavin Deficiency.

     The clinical assessment of riboflavin nutriture in man is
inexpensive, can be done quickly and requires minimal training.
There are, however, several problems in the clinical evaluation of
nutritional status such as examiner variation that must be borne
in mind at all times (Plough and Bridgeforth, 1960). It is rare to
find pure, uncomplicated riboflavin deficiency in clinical practice.
Most patients with ariboflavinosis will also be deficient in other
nutrients which may alter riboflavin status. For example, it has

been stated that deficiencies of pyridoxine and niacin cause the
same lesions of the mouth typically associated with riboflavin
deficiency (Goldsmith, 1956; Jelliffe, 1966; Krishnaswamy, 1971;
Iyenger, 1973). The rationale for this statement is discussed in
the section on Pathophysiology.

It is also important to realize that clinical manifestations
are late reflections of metabolic disturbances that occur only
after nutrient body stores have become depleted. Brin et al.
(1965) have described the progressive stages of vitamin deficiency
in man. Most nutrition surveys done in the United States reveal
a rather low incidence of clinical evidence of riboflavin de-
ficiency. This should not be taken as an indication that sub-
clinical ariboflavinosis is not present. Clinical observations
should only be utilized as a quick screening method to identify
those patients at greatest risk of being riboflavin-deficient.
Confirmation by dietary evaluation and biochemical assessment is
absolutely necessary to ensure proper treatment.

Biochemical

There are several biochemical methods for measuring riboflavin
and guidelines for interpretation of the results (Pearson, 1962,
1967a; O'Neal et al., 1970; Sauberlich et al., 1974). Biological
and chemical assays can be used with samples of tissue, urine or
blood. These tests measure changes reflecting dietary riboflavin
intake or tissue storage and saturation of riboflavin, FMN and FAD
(Kaufmann and Guggenheim, 1977). Other analytical techniques have
been described but are not used often (Couch and Davies, 1963).
Recently, an enzymatic functional test to assess riboflavin status
has been developed which appears to be more valid than other
measurements. All of these assays are sensitive to small changes
in experimental conditions but are reproducible and accurate when
done by capable and experienced laboratory personnel.

Sample Types

Tissue. When we attempt to measure riboflavin status, we
are, in effect, seeking a measure of body stores (Pearson, 1967a).
These stores are not markedly affected by normal dietary riboflavin
intakes because they are not mobilized until the circulating ribo-
flavin in blood has been depleted (Tillotson and Baker, 1972).
Thus, tissue flavin levels are an ideal measurement. Most of the
riboflavin in tissues is present as FMN and FAD bound to protein.
These flavins must be extracted, liberated and separated prior to
analysis according to any of several published procedures (Yagi,
1951, 1962; Yagi and Okuda, 1958; Pearson, 1967b; Koziol, 1971;
Fazekas and Kokai, 1971; Baker and Frank, 1975). The release of
covalently-bound flavin, e.g., FAD present in succinic dehydro-
genase, requires enzyme and acid digestion for liberation

(Singer et al., 1971). Tissue measurements are rarely made in man because they are very difficult to obtain and gram quantities of tissue are required.

Urine. Riboflavin status has been evaluated extensively by measuring its levels in urine (Horwitt, 1966). For all practical purposes the only flavin present in urine is riboflavin. Despite the lack of human data on the relationship between urinary excretion and total body stores of riboflavin (Pearson, 1967a), the clinical signs of ariboflavinosis and dietary intake of the vitamin have been shown to correlate with urinary riboflavin levels under carefully controlled studies (see Requirements Section).

For survey purposes non-fasting casual, 2-hour or 6-hour collections can be used successfully (Lowry, 1952; Hegsted et al., 1956; Plough and Consolazio, 1959). In clinical instances where riboflavin deficiency is suspected, a 24-hour urine collection should be obtained if at all possible (Sauberlich et al., 1974). Expressing urinary riboflavin excretions per gram of creatinine tends to correct for variations due to body size. When this correlation is applied, children excrete more riboflavin per gram of creatinine than do adults (Pearson, 1962). A riboflavin load test had been used occasionally in which the urinary excretion of riboflavin is measured in the 4-hour period following the oral administration of a 5-mg test dose of the vitamin (Lossy et al., 1951). Several considerations must be taken into account when using urine to assess riboflavin nutriture.

Urinary riboflavin levels give no indication of the body's stores of FMN and FAD, the functional flavin forms. There can be large variations in urinary excretion due to differences in dietary intake (Tillotson and Baker, 1972) and physiological circumstances. Riboflavin excretion is increased under conditions of negative nitrogen balance, fasting, heat, stress, prolonged hard physical work and enforced bed rest (Pollack and Bookman, 1951; Bro-Rasmussen, 1958; Smith et al., 1959; Tucker et al., 1960; Coon, 1965). Sleep and short periods of hard physical work decrease vitamin excretion (Tucker et al., 1960).

Blood. Riboflavin nutritional status has also been evaluated by measuring riboflavin, FMN and FAD levels in whole blood, plasma and erythrocytes (Pearson, 1962). As was the case with urine, plasma concentrations of flavins tend to reflect recent dietary intake and exhibit considerable daily fluctuation (Burch et al., 1948; Suvarnakich et al., 1952; Coon, 1965; Bamji, 1969). Erythrocyte flavin levels are more sensitive than plasma and have been used in several studies as an index of riboflavin status (Horwitt et al., 1949; Bessey et al., 1956; Bamji, 1969; Clarke, 1969b). Blood levels are, nevertheless, of limited value since concentrations do not change until more easily determined parameters have

been altered (Sauberlich et al., 1974).

Assay Methods

Fluorometric. Most fluorometric assays are based on the
method of Slater and Morell (1946). Procedures are available for
use with tissues (Bessey et al., 1949; Burch et al., 1956), blood
(Burch et al., 1948) and urine (Albright and Degner, 1967; Mellor
and Maass, 1972). Either the fluorescence of the flavins can be
measured directly or they can be converted to lumiflavin and its
fluorescence determined (Fujita and Matsuura, 1950; Clarke, 1969a,
1977a). Fluorometric methods of analysis are used more often
than microbiological procedures as they tend to give higher and
more consistent values (Bamji et al., 1973).

Biologic. Aside from the lactic acid bacteria, few free-
living bacteria require exogenous riboflavin. Optimal assay con-
ditions for using Lactobacillus casei to determine flavin levels
are well documented (Snell and Strong, 1939; Strong and Carpenter,
1942; Clegg et al., 1952; Prager et al., 1958). Growth of the
Lactobacillus casei can be measured turbidimetrically or by the
level of acid production. Although not used as frequently,
Leuconostoc mesenteroides is claimed to be 50 times more sensitive
than L. casei for assaying flavins (Kornberg et al., 1948). There
are, however, certain disadvantages in using microbiological assays.
Antibiotic therapy in patients often precludes their use. The
growth response of L. casei is different for riboflavin, FMN and
FAD (Snell and Strong, 1939; Langer and Charoensiri, 1966). L.
casei is also known to grow in response to fatty acids (Pearson,
1962) and flavin analogs that may or may not have biologic activity
(Yang et al., 1964). Microbiological assays tend to be more
satisfactory for urine determinations than they are for blood
measurments (Pearson, 1967b). A method based on the protozoan,
Tetrahymena pyriformis, has been successfully used by Baker et al.,
(1966) for several years.

Enzymatic. In recent years EGR has been used as an enzymatic
index of the riboflavin nutriture of man. The enzyme catalyzes
the reduction of oxidized glutathione (GSSG) in the following
manner:

$$NADPH + H^+ + GSSG \xrightarrow{\quad FAD \quad} NADP^+ + 2GSH$$
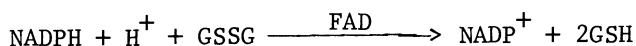
EGR activity is altered in vivo by dietary riboflavin (Bamji, 1969;
Beutler, 1969; Glatzle et al., 1970) and in vitro by FAD using
purified enzyme (Buzard and Kopko, 1963; Scott et al., 1963; Icen,
1967; Staal et al., 1969) and red cell hemolysates (Glatzle et al.,
1968; Beutler, 1969; Glatzle et al., 1970). The degree of in
vitro stimulation of EGR activity depends on the degree of apo-
enzyme saturation by FAD, which in turn depends upon the

availability of riboflavin. The enzyme is usually not saturated
with FAD (Beutler, 1969), but a relatively constant percentage of
FAD saturation of the EGR is found in persons consuming adequate
amounts of riboflavin (Tillotson and Baker, 1972). A direct
measurement of EGR activity can be used to determine riboflavin
status, but to do so requires a carefully standardized assay proce-
dure and the use of a reference such as hemoglobin to eliminate
the effects of factors such as anemia. Much more frequently EGR
activity is measured in the presence and absence of in vitro FAD.
This stimulatory effect is then expressed as an activity coeffi-
cient - EGR AC.

$$\text{EGR AC} = \frac{\text{Enzyme Activity with added FAD}}{\text{Enzyme Activity without added FAD}}$$

The question of what specific assay conditions give maximal
EGR activity has received considerable attention (Staal and Veeger,
1969; Staal et al., 1969; Hornbeck and Bradley, 1974; Schorah and
Messenger, 1975; Bayoumi and Rosalki, 1976). Enzyme activity can
be measured by following the oxidation of NADPH at 340 mμ (Beutler,
1969; Glatzle et al., 1970; Brewster et al., 1974; Nichoalds et al.,
1974) or the production of GSH (Tatt et al., 1975; Garry and Owen,
1976). Whole blood (Weber et al., 1973; Glatzle et al., 1974) and
white blood cells (Muller and Bates, 1977) can be used instead of
red cells.

The EGR AC test has numerous advantages. It requires only
small amounts of blood and is independent of age and sex. Fasting
blood samples are not needed and the enzyme is relatively stable.
The usefulness of the test for identifying individuals receiving
marginal dietary riboflavin over prolonged periods of time as well
as individuals suffering from severe riboflavin deficiency has been
well documented (Thurnham et al., 1970, 1972; Buzina et al., 1971;
Flatz, 1971a,b,c; Tillotson and Baker, 1972; Sauberlich et al.,
1972, 1974; Cooperman et al., 1973; Weber et al., 1973).

While the EGR AC responds rapidly to riboflavin deficiency,
it plateaus quickly and does not continue to change in response to
progressive tissue depletion (Sterner and Price, 1973). The test
cannot be used in persons with G-6-P-D deficiency due to a greater
avidity of EGR for FAD (Flatz, 1970; Thurnham, 1972); the preva-
lence of G-6-P-D deficiency is about 10% among American Blacks
(Schrier et al., 1958; Frischer et al., 1973). Other diseases may
also alter EGR activity in a manner unrelated to riboflavin status
(Beutler, 1969; Yawata and Tanaka, 1971) and more information in
this area is needed.

At the moment the measurement of the EGR AC appears to be the
method of choice for evaluating riboflavin nutriture in man. Some
of the areas which certainly warrant closer investigation include

luminescence and pyridoxamine 5'-phosphate (PMP) oxidase. The
application of the luciferase reaction (Strehler and Cormier, 1954)
in the clinical laboratory has recently been reviewed (Gorus and
Schram, 1979; Whitehead et al., 1979) and both FMN and FAD can be
measured using this technique (Mitchell and Hastings, 1969;
Chappele and Picciolo, 1971; Stanley, 1971; Meighen and MacKenzie,
1973). PMP oxidase activity has been found to decrease signifi-
cantly in liver, kidney and brain tissue of riboflavin-deficient
rats (Rasmussen et al., 1979). Importantly, PMP oxidase activity
is more sensitive than the ERG AC to the degree of riboflavin
depletion.

OCCURRENCE OF RIBOFLAVIN DEFICIENCY

     Riboflavin status can be evaluated by assessing the adequacy
of dietary intake, the presence or absence of clinical signs
associated with ariboflavinosis, and by several different biochem-
ical determinations of riboflavin levels in the body. Using one
or more of these criteria, indications of inadequate riboflavin
nutriture have commonly observed in nutrition surveys (Davis
et al., 1969; Kelsay, 1969; May, 1961-1972; Ten-State Nutrition
Survey, 1968-1970).

     The largest series of international nutrition surveys were
conducted by the Interdepartmental Committee on Nutrition for
National Defense. The incidence of riboflavin deficiency is ex-
tremely high in some of the developing countries such as India,
Thailand and Trinidad. In situations where a population subsists
on insufficient quantities of food which are poor sources of ribo-
flavin, over 25% of the people may suffer from riboflavin de-
ficiency (Fernandez et al., 1965). The frequency of ariboflavinosis
is seasonal in developing countries and can vary 10-fold (Thurnham
et al., 1970; Buzina et al., 1973).

     Except for particularly vulnerable segments of the American
population such as patients with disease, riboflavin deficiency is
an unexpected entity in a country such as the United States where
an abundance of food is available. There is, however, a propensity
of riboflavin deficiency in the socio-economically deprived groups
of the population (Ten-State Nutrition Survey, 1968-1970) which has
been attributed to the poor quality of dietary intake. Potential
health problems are particularly manifested among impoverished
Blacks and Spanish Americans of all ages. It has been pointed out
earlier (Leverton, 1964) that the nutritive value of a nation's
food supply cannot be used to assess the nutritional status of an
individual.

     Ariboflavinosis often develops as a consequence of pregnancy
(Brzezinski et al., 1952). Oral lesions of the mouth associated
with inadequate riboflavin nutriture are commonly observed in

malnourished pregnant women (Clarke, 1971; Iyengar, 1973; Bamji, 1976; Clarke, 1976). Some investigators have not found riboflavin deficiency to be a problem in pregnancy (Baker et al., 1975), but the largest survey done to date reported an incidence of riboflavin depletion among apparently healthy women of up to 40% using the EGR AC test (Heller et al., 1974). Generally, the frequency of riboflavin deficiency increases with the progress of pregnancy.

Pregnant adolescents are the most vulnerable segment of the population to generalized malnutrition. Data on American teenagers are limited but indicate rates of riboflavin deficiency ranging from 10% to 30% (McGanity et al., 1969; Kaminetzky et al., 1973). In view of the recent tremendous increase in the occurrence of teenage pregnancy in this country (Stickle, 1975), more nutritional studies on this particular group are direly needed.

The Ten-State Nutrition Survey (1968-1970) identified riboflavin deficiency as a potential problem for infants and children. Preschool children in Mississippi (Owen et al., 1969) and children under 12 years of age from both lower and upper income groups in New York City (Newman and Martin, 1971) had unacceptable urinary riboflavin values ranging from 6 to 9%. In a more recent survey (Lopez et al., 1975) 11 out of 100 children ranging in age from a few days to 14 years who resided in economically depressed areas of New York City had biochemical evidence of inadequate riboflavin nutriture.

Undesirably low levels of riboflavin in the urine of teenagers have been reported to occur at a rate of 7% in Iowa (Hodges and Krehl, 1965), 5% in Vermont (Morse et al., 1965) and 21% in New York (Dibble et al., 1965). Sauberlich et al. (1972) evaluated the riboflavin nutriture of 431 rural Tennessee high school students by several criteria: 1) 47% of the adolescents had dietary riboflavin intakes less than 50% of the National Research Council's Recommended Dietary Allowance (RDA, 1980); 2) unacceptable urinary excretion of riboflavin was found in 22% and; 3) the incidence of inadequate riboflavin nutriture was 11% using the EGR AC assay. In all these studies riboflavin deficiency was more common in girls and probably reflects greater food consumption by the boys.

Information concerning riboflavin status in the aged is meager (Department of Health and Social Security, 1972) and has resulted in contradictory conclusions. Evaluations of riboflavin nutriture by measuring whole blood levels or urinary excretion have indicated a rather low incidence of deficiency (Brin et al., 1965; Brocklehurst et al., 1968; Thackray et al., 1972; Morgan et al., 1975) in elderly subjects despite a relatively high incidence of clinical signs associated with ariboflavinosis. This discrepancy is likely due to the insensitivity of whole blood measurements as is borne out by the observation that riboflavin supplementation does

alleviate the clinical situation (Dymock and Brocklehurst, 1973).
Failure to confirm the positive therapeutic effects of riboflavin
(Berry and Darke, 1972) probably means that factors other than
riboflavin deficiency caused the observed clinical signs and
symptoms (see Clinical Signs and Symptoms Section). When ribofla-
vin status has been assessed using the EGR AC technique, deficiency
in geriatric subjects has been found to occur in 7% to 32% of this
age group (Glatzle et al., 1970; Hoorn et al., 1975; Kaufmann and
Guggenheim, 1977; Vir and Love,1977).

Diseases can precipitate and/or exacerbate riboflavin de-
ficiency. Ariboflavinosis has been observed in prolonged febrile
illnesses such as rheumatic fever, tuberculosis and sub-acute
bacterial endocarditis, in malignancy, hyperthyroidism, cardiac
failure, diabetes mellitus and diseases of the gastroentestinal
tract (Goldsmith, 1975). Table II summarizes some of the diseases
reported to aggravate riboflavin nutriture.

Perhaps the most common disease known to contribute to mal-
nutrition in developed countries is alcoholism. Chronic consumption
of relatively large quantities of alcohol can result in riboflavin
deficiency. Low blood levels of riboflavin have been found to occur
in 15% of alcoholics having no liver damage (Leevy et al., 1965a).
Those with fatty livers have essentially the same incidence of de-
ficiency (17%) but the prevalance increases to 25% in those with
cirrhosis. A second complicating illness superimposed on the alco-
holism can increase the rate of riboflavin deficiency to 50%
(Rosenthal et al., 1973).

Recent reports (Butterworth, 1974; Butterworth and Blackburn,
1975) have presented rather alarming evidence that many hospital-
ized patients suffer from malnutrition, some of which is acquired
after being hospitalized. Significant episodes of riboflavin de-
ficiency have been found in patients in hospitals (Brocklehurst
et al., 1968; Vir and Love,1977; Tanphaichitr et al., in press).
In a random sample of 120 hospital patients in New Jersey, 12% had
low levels of riboflavin in their blood (Leevy et al., 1965b).

CAUSES OF DEFICIENCY

The causes of ariboflavinosis can be delineated as being due
to: 1) inadequate intake; 2) decreased assimilation; 3) defective
utilization; 4) increased requirement; 5) increased destruction
and 6) increased excretion. The following examples of causes of
riboflavin deficiency are applicable to nutrients in general.

Good riboflavin nutriture is initially dependent upon the
availability of riboflavin-rich foods and then upon the capability
to obtain those foods in sufficient quantities for consumption.
In developing countries there is often a paucity of food for reasons

Table II. Riboflavin Deficiency in Disease

Disease	References
Cancer	Rivlin, 1975a
Cardiac	Steier et al., 1976
Diabetes mellitus	Pollack and Brookman, 1951 Cole et al., 1976
Gastrointestinal and Biliary Obstruction	Jusko and Levy, 1967 Rivlin, 1970b Jusko et al., 1971 Lai and Ransome, 1971 Tautt, 1976 Rosenberg et al., 1977 Sleisenger and Brandborg, 1977 Hines, 1978
Infection	Beisel et al., 1972 Feigin, 1977
Liver	Baker et al., 1964 Leevy et al., 1965a Clarke, 1969b Leevy et al., 1970 Rosenthal et al., 1973 Mezey, 1978
Thyroid	Rivlin, 1968, 1970a, 1970b, 1975b, 1979

of nature such as drought, floods and insect infestation. However, in developed countries such as the United States, the difficulty of being able to purchase an adequate diet is much more of a problem than availability of food.

Inadequate riboflavin consumption may also be the result of food habits, traditions, customs and religious beliefs which lead to the avoidance of riboflavin-rich foods. Pica is one example of a relatively prevalent food habit (Kaminetzky et al., 1973) that can reduce riboflavin intake. One of the more popular and less radical food faddism movements in the United States has been vegetarianism. Most evaluations indicate that vegetarian diets can be adequate if sufficient thought goes into their planning (Register and Sonnenberg, 1973). In fact women on vegetarian diets have been reported to undergo pregnancy without any adverse effects on themselves or their offspring (Thomas and Ellis, 1977). Many traditional dietary restrictions apply most often to the nutritionally vulnerable groups - infants, toddlers, pregnant women, lactating women, and the sick. In addition, if anorexia is present, there can be a substantial decrease in food intake.

Commonly employed food processing techniques and storage practices can result in appreciable losses of riboflavin (Foy and Mbaya, 1977). Even cooking habits can decrease the availability of riboflavin present in food. Extensive riboflavin losses of up to 20% occur when foods are cooked for long periods of time in large volumes of water which are subsequently discarded (Krehl and Winters, 1950).

Ineffective digestion and/or absorption of food results in diminished assimilation. Defective digestive enzymes can also dramatically reduce nutrient assimilation. A lucid example found in most Asian people and Blacks is lactase deficiency which leads to an intolerance of milk, a particularly good source of riboflavin. Lactase is often the first digestive enzyme to be affected during illnesses such as diarrhea and protein-calorie malnutrition.

Conditions that cause increased motility and decreased gastrointestinal passage time can also result in malassimilation. Exemplary illustrations of some of these conditions include ingestion of laxative agents, antibiotic therapy, infectious enteritis, blockage of the biliary pathway and certain diseases such as irritable bowel syndrome and malabsorption syndrome (see Table II). Antibiotics may also cause riboflavin deficiency by altering the intestinal flora and inhibiting bacterial synthesis of riboflavin (Tuszynsky et al., 1958).

Malabsorption occurs whenever there is appreciable structural damage and/or a decrease in mucosal cells of the small intestine. This can occur as the result of ingestion of caustic agents,

maladies such as tropical sprue and celiac disease, malignancy and resection of the small bowel (Hines, 1978). Finally, systemic diseases without primary involvement of the gastrointestinal tract, such as chronic liver disease and systemic bacterial infection, can also result in failure of riboflavin absorption (Jusko and Levy, 1967; Feigin, 1977).

Once riboflavin is present inside the body, the vitamin may not be maximally utilized. This can be caused by disturbances in hormonal production, e.g. thyroid, ACTH and aldosterone (Rivlin, 1979) as well as insulin. Certain drugs such as oral contraceptive pills (Briggs and Briggs, 1974; Sanpitak and Chayutimonkul, 1974; Ahmed et al., 1976b; Newman et al., 1978) and phenothiazine derivatives (Gabay and Harris, 1966, 1967; Horvath, 1976) also appear to impair riboflavin utilization.

The body's need for riboflavin becomes greater during the periods of physiological stress that occur during growth, pregnancy and lactation. Since physiological stress is usually construed as being normal, its importance is frequently neglected or underestimated in clincial practice. Pathological stress due to diseases can also be expected to increase nutrient requirements but, this subject has received little attention and definitive data are not available.

The treatment of neonatal jaundice with phototherapy has been observed to result in riboflavin deficiency by causing accelerated decomposition of the vitamin (Rubaltelli et al., 1974; Gromisch et al., 1977). Indirect evidence of riboflavin deficiency has similarly been noted in patients with β-thalassemia, another hematologic disease, during hemolytic episodes (Anderson et al., 1976).

Increased riboflavin excretion occurs in all conditions which produce increased nitrogen excretion, the cause of which may be attributed solely to the catabolic process of disease such as burns, uncontrolled diabetes mellitus, hyperthyroidism, systemic infections, starvation or even simple bed rest (Bro-Rasmussen, 1958; Tucker et al., 1960; Clarke, 1969b; Beisel et al., 1972; Feigin, 1977). Increased excretion of riboflavin can also be caused by certain antibiotics and phenothiazine drugs used in treatment processes (Goldsmith, 1975; Pinto et al., 1979).

The foregoing clearly points out that the etiology of riboflavin deficiency may range from simple inadequate dietary intake to complex and multiple causes, complicated by disease. For example, protein-calorie malnutrition diminishes both riboflavin absorption and utilization. The ariboflavinosis seen in alcoholics may be due to generally poor dietary intake or specific effects of alcohol upon the absorption, metabolic transformations or excretion of this

vitamin. Systemic infections, even without gastrointestinal tract
involvement, can cause inadequate intake, defective absorption,
increased requirement, poor utilization and increased excretion.

EFFECTS OF RIBOFLAVIN DEFICIENCY

Clinical Signs and Symptoms

     Since the first report of human riboflavin deficiency (Sebrell
and Butler, 1938), there have been many experimental studies to
elucidate the clinical symptoms and signs associated with aribo-
flavinosis. The following sections describe the known clinical
manifestations of riboflavin deficiency in man. Other clinical
descriptions include those by Jolliffe et al. (1939), Brocklehurst
et al. (1968) and Goldsmith (1975).

     Oral Mucosal and Tongue Alterations. Early symptoms of ribo-
flavin deficiency include soreness of the lips, tongue and mouth
(Lane et al., 1964) and are usually accompanied by difficulty in
eating and swallowing. Pallor develops at the lip mucosa in the
angles of the mouth followed by maceration. The lesions are bi-
lateral and will be covered by a honey-colored crust. They extend
downwardly from the angles of the mouth to the normal skin produc-
ing superficial transverse fissures known as angular stomatitis
or perleche in the older literature. Any lesion resembling angular
stomatitis that occurs only on one side is probably due to infection
from herpes simplex rather than being attributable to riboflavin
deficiency. Ill-fitting dentures can also cause similar lesions
(Goldsmith, 1959), particularly in the elderly (Exton-Smith, 1975).
At about the same time the lips may become swollen and denudation
of the mucosa along the closure line of the mouth often occurs
causing vertical fissures which are called cheilosis. These
lesions may be the result of exposure to weather and have been
found in persons with combined niacin and pyridoxine deficiency
(Mueller and Vilter, 1950).

     Glossitis is often seen in riboflavin-deficient persons. In
the early stages the tongue which is normally pink in color becomes
red accompanied by swelling and a burning sensation. It may be
deeply fissured with prominent fungiform papillae at the tip. The
patient may experience sore throat. As the riboflavin deficiency
worsens, the filiform papillae begin shedding in small patches
which gives the tongue a pebbled appearance (Figure 3). In the
next progressive stage the filiform papillae continue shedding
until the tongue becomes smooth (Figure 4). Taste acuity is dimin-
ished at the same time. If the patient does not receive treatment,
the tongue will finally become atrophic. Glossitis is common in
deficiency of many of the B-complex vitamins.
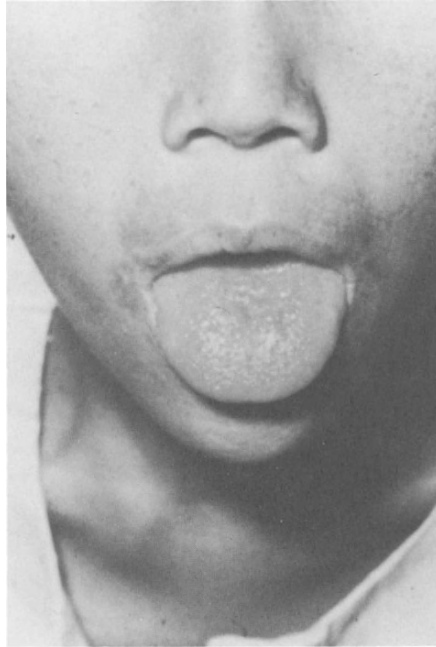
Fig. 3.   Early stage of angular stomatitis. Note the patchy
          shedding of the filiform papillae and prominent
          fungiform papillae producing a pebbled appearance
          of the tongue.

Ocular Changes.   Ocular changes due to riboflavin deficiency
were first noted in man by Spies et al. (1939). Sydenstricker
et al. (1940) beautifully described the progress and regress of
the occular manifestation due to ariboflavinosis in patients.
Photophobia and blepharospasm are always the first eye symptoms
followed by conjunctival conjestion. The patients experience a
burning sensation of the eyeballs and a dimness of vision uncorrec-
table by adjustment of the refractive errors. Circumcorneal in-
jection can be grossly visualized and is usually accompanied by
conjunctivitis. Examination by ophthalmoscopes and slit lamps is
helpful in the early diagnosis of the disorder. In severe in-
stances, capillary invasion from the circumcorneal plexus into the
cornea will be observed. The capillary growth into the normally
avascular cornea is probably an attempt to compensate for the break-
down of oxidation processes in the corneal cells (Bessey and
Wolbach, 1939). Occasionally, frank iritis can be seen. It is
noteworthy that comparable abnormalities can also be found in
association with trauma, infection and allergic conditions. Some
animal studies have implicated riboflavin deficiency as a causative

Fig. 4. Advanced stage of angular stomatitis and glossitis.
         Note the smooth tongue with prominent fugiform
         papillae.

factor in cataract formation (Day et al., 1931; Eckardt and Johnson,
1939; Srivastava and Beutler, 1970) but such a relationship has
not been demonstrated in man (Nutr. Rev., 1976).

Dermatological Changes. Another common clinical manifestation
of ariboflavinosis in man is seborrheic dermatitis. Frequently
there is a greasy desquamation in the facial area that occurs in
the naso-labial fold, alae nasi, nasal vestibule and on the ears.
Sometimes the skin around the eye lids near the inner and outer
canthi is also involved and is called angular palpebritis.
Seborrheic dermatitis may occur at the scrotum in males and at the
vulva in females (Horwitt et al., 1949). Similar changes often
occur in the adjacent areas of the thighs characterized by redness,
scaling, desquamation and oozing of serum from the superficial skin.
Seborrheic dermatis may also be related to a lack of pyridoxine.

The interpretation of the above dermatological lesions is
complicated by the appearance of similar lesions that have been
described in patients with zinc deficiency. Particular attention
has been drawn to patients who have received long-term total

parenteral nutrition unsupplemented with zinc (Kay et al., 1976; Okada et al., 1976). In addition to dietary zinc deficiency, there is a genetically inherited disorder seen in infants and young children called acrodermatitis enteropathica that also re- sults in zinc deficiency (Moynahan, 1974) and gives rise to derma- tological lesions. However, there appears to be no metabolic interaction between riboflavin and zinc deficiencies (Pallauf and Kirchgessner,1973).

Neurological Alterations. Burning feet syndrome has been reported in one patient diagnosed as having malabsorption; the sensory abnormalities were alleviated within one week by intra- muscular injections of riboflavin (Lai and Ransome, 1970). A decrement of hand grip strength has been noted in ariboflavinosis (Sterner and Price, 1973). Retarded intellectual development and electro-encephalographic changes have been observed in riboflavin- deficient children (Arakawa et al., 1968a). In adults, experimental hyporiboflavinosis may produce behavioral changes such as hypo- chondriasis, depression, hysteria, psychopathic-deviate and hypo- mania (Sterner and Price, 1973). The effects of dietary deprivation on human behavior have been reviewed by Brozek and Vaes (1961).

Hematological Dyscrasia. A few studies have indicated an indirect role for riboflavin in hematological dyscrasia. Normocy- tic-normochromic anemia and reticulocytopenia have been induced in man using a dietary regimen that was not only riboflavin-deficient but also contained a riboflavin antagonist (Lane and Alfrey, 1965). Similar hematological findings have also been found to occur in African children with marasmus and kwashiorkor (Foy et al., 1961; Kondi et al., 1967). All symptoms of the hematological dyscrasia that occur in riboflavin-deficient adults, and in children with protein-calorie malnutrition, regress after treatment with either riboflavin or corticosteroid, suggesting the possibility of an interrelationship between riboflavin deficiency, corticosteroids and the hematopoietic system. Details concerning the pathophysio- logy of these abnormalities are discussed in the Pathophysiology Section.

Pathophysiology

Much recent data have clearly shown that riboflavin nutriture in man is influenced by numerous factors. At the same time it is also evident that riboflavin deficiency alters several metabolic pathways. Figure 5 summarizes the currently recognized interrela- tionships of riboflavin with other nutrients and the hormonal con- trol of its conversion to active coenzymes. The following examples of the pathophysiology of riboflavin deficiency are not intended to constitute an exhaustive review of the literature but will serve to highlight major areas of concern where some data are available.
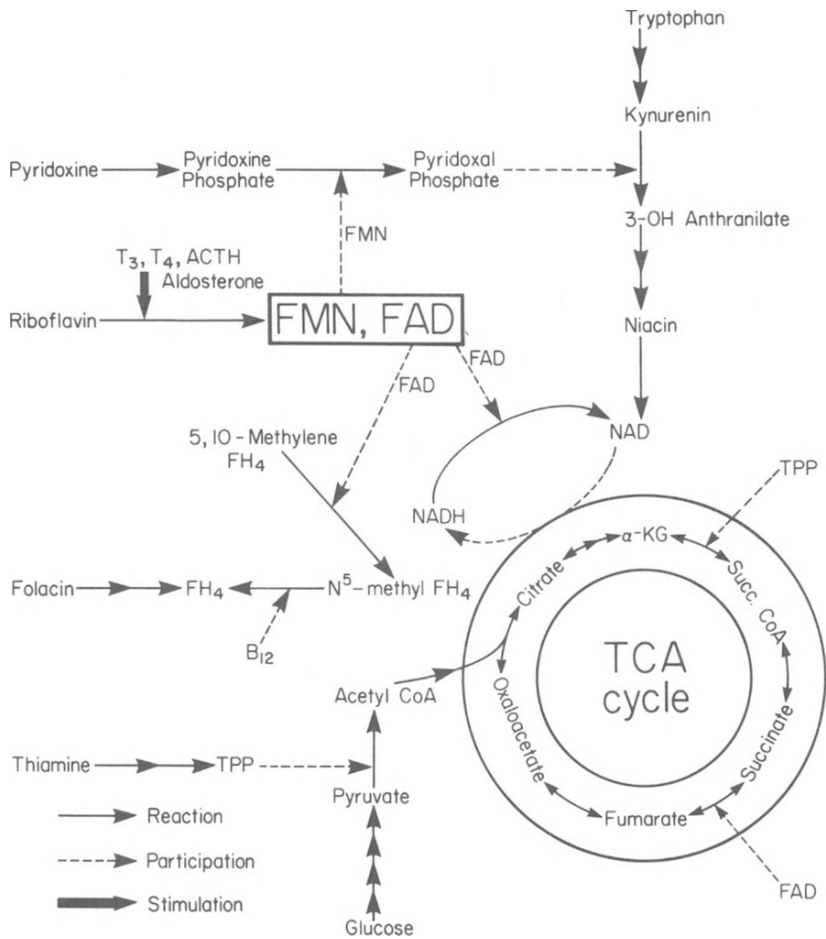
Fig. 5.   Interrelationships of riboflavin with other
          nutrients and hormonal control.

Hormonal Control.   The role of hormones in controlling ribo-
flavin metabolism has just recently been reviewed (Rivlin, 1979).
Several lines of evidence suggest that thyroid hormones augment
flavokinase and FAD pyrophosphorylase activities (Rivlin et al.,
1968; Rivlin, 1970a,b, 1975b), thereby enhancing the conversion
of riboflavin to FMN and FAD.   Changes in the activities of the

above two enzymes and flavin coenzyme levels during hypothyroidism
further support the concept that thyroid hormones play a role in
controlling riboflavin metabolism. This control may extend to that
fraction of flavins bound covalently to tissue proteins (Rivlin,
1979). This interaction is cyclic in that riboflavin deficiency
impairs the synthesis of thyroid hormones.

ACTH and aldosterone have also been shown to enhance the
formation of FMN and FAD in adrenal cortex, liver and kidney
(Fazekas and Sandor, 1971, 1973; Tan and Trachewsky, 1975;
Trachewsky, 1978). As is the case with thyroid hormones, aribo-
flavinosis diminishes steroid biosynthesis by the adrenal glands.
Additional research is needed to clarify the consequences of
hormonal abnormalities concerning riboflavin metabolism.

Vitamin Interrelationships. The clinical signs and symptoms
of ariboflavinosis have often been associated with pyridoxine and
niacin deficiencies (Spies et al., 1939; Mueller and Vilter, 1950;
Vilter et al., 1953; Krishnaswamy, 1971; Iyengar, 1973; Lakshmi
and Bamji, 1974). Patients diagnosed with clinical and biochemical
riboflavin deficiency have been noted to respond positively to
pyridoxine supplementation (Mueller and Vilter, 1950; Iyengar,
1973) even though such therapy does not improve the EGR AC (Lakshmi
and Bamji, 1974). The interrelationships of riboflavin, pyridoxine
and niacin are summarized in Figure 5.

Riboflavin is necessary for the aerobic conversion of pyri-
doxine to pyridoxal phosphate (PLP), the active coenzyme form, as
shown in the following reaction (Wada and Snell, 1961; Sharada and
Bamji, 1972; Anderson et al., 1976).

$$\left.\begin{array}{l}\text{Pyridoxamine } 5'PO_4 \\ \text{Pyridoxine } 5' \ PO_4\end{array}\right] - \quad \xrightarrow[\text{PMP Oxidase}]{\text{FMN}} \quad \text{Pyridoxal } 5'PO_4$$
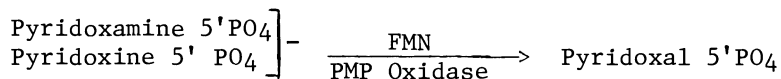
PMP oxidase has been postulated to be a key control point in pyri-
doxine metabolism (Merrill et al., 1978). Riboflavin deficiency
causes a reduction in PMP oxidase activity which in turn results
in diminished conversion of pyridoxine to PLP (Lakshmi and Bamji,
1974, 1976). In very pronounced riboflavin deficiency there is an
accumulation of pyridoxine in the liver but liver and blood levels
of PLP are unaffected. The latter may be due to decreased PLP
phosphatase activity or there may be a FMN-dependent pathway for
the formation of PLP (Lakshmi and Bamji, 1974, 1976).

By virtue of its role in the production of PLP, riboflavin
is also important in tryptophan metabolism and the formation of
NAD. Riboflavin deficiency causes an increased urinary excretion
of tryptophan metabolites (Mason, 1953; Verjee, 1975; Rose and
McGinty, 1968). Depleted body stores of niacin found in pella-
grins show improvement with riboflavin therapy (Spies et al., 1939).

All of these findings are consistent with the suggestion that a
cellular deficiency of PLP is the basis of the clinical lesions
(cheilosis and angular stomatitis) seen in ariboflavinosis
(Sharada and Bamji, 1972).

Numerous investigations point towards a close metabolic link
between thiamin and riboflavin. In thiamin deficiency there is an
increased urinary excretion of riboflavin (Tucker et al., 1960)
and an increased accumulation of riboflavin in the liver (Singher
et al., 1944; Bamji and Sharada, 1972; Sharada and Bamji, 1972).
In similar fashion riboflavin deficiency results in enhanced liver
thiamin levels (Singher et al., 1944). Thiamin supplementation of
rats fed diets deficient in both thiamin and riboflavin increases
EGR activity and decreases the EGR AC value (Vo-Khactu et al.,
1976). Thus, it appears that a deficiency of either vitamin will
alter the utilization of the other vitamin (see Figure 5).

Interactions between riboflavin and folacin also exist. Ribo-
flavin deficiency causes derangements in folic acid metabolism in
tissue, blood and urine (Miller et al., 1962; Foy et al., 1966;
Honda, 1968; Narisawa et al., 1968; Bovina et al., 1969). A number
of enzymes involving folic acid are also diminished in activity
(Arakawa et al., 1968b; Narisawa et al., 1968). These derangements
in folacin metabolism are probably attributable to reduced activity
of flavoprotein enzymes required for normal utilization of folic
acid (Honda, 1968; Narisawa et al., 1968). Folacin deficiency, on
the other hand, has no effect on blood levels of riboflavin or on
EGR AC values (Bamji and Sharada, 1972).

Serum levels of vitamin B_{12} remain unchanged or are elevated
during ariboflavinosis (Lane et al., 1964; Foy et al., 1966; Foy
and Kondi, 1968). The increased blood levels of vitamin B_{12} return
to normal following riboflavin administration and likely reflect
decreased utilization of vitamin B_{12}.

General Metabolic Derangements. Flavins are involved in a
variety of oxidation-reduction processes in living cells and are
particularly important in the utilization of carbohydrates, lipids
and proteins (Foy and Mbaya, 1977). Many of the effects of ribo-
flavin deficiency can be explained by abnormalities in hormonal
control of riboflavin metabolism and interaction with other vitamins
as just discussed. Forker and Morgan (1954) noted impaired gluco-
neogenesis in riboflavin deficiency which was normalized by ribo-
flavin supplementation. Such changes in gluconeogenesis accompany
a number of enzymatic and metabolic disturbances and, although
important, cannot be regarded as specific and are probably only
one of several different responses the body makes to a variety of
stresses (Garthoff et al., 1973). There is at the same time an in-
creased accumulation of liver glycogen (Chatterjee and Ghosh, 1967)
during ariboflavinosis, some of which can be attributed to enhanced

alanine transaminase activity and conversion of alanine to glycogen (Chatterjee et al., 1966). Increased glycogen stores may also, in part, be the result of diminished glycolysis due to reduced TCA cycle metabolism and decreased utilization of thiamin.

Lipid metabolism is particularly sensitive to riboflavin status. A deficiency of the vitamin prevents the beta-oxidation of fats and inhibits their utilization. This results in a greatly increased liver content of triglycerides (Burch et al., 1956; Sugioka et al., 1969). A reduction in the dehydrogenation of fatty acids occurs after prolonged riboflavin deficiency and causes diminished levels of linoleic, linolenic and arachidonic acids in serum and liver (Mookerjea and Hawkins, 1960; Koyanagi and Oikawa, 1965). Pronounced decreases in fatty acid oxidation occur prior to the morphological changes in hepatic mitochondrial cristae induced by riboflavin deficiency (Tandler et al., 1968; Hoppel and Tandler, 1976).

Protein metabolism is also affected by riboflavin deficiency. When dietary levels of protein are inadequate, the body cannot retain riboflavin and it is excreted in the urine (Sarette and Perlzweig, 1943; Bro-Rasmussen, 1958). The ensuing low body levels of riboflavin in turn result in deranged amino acid metabolism (see Vitamin Interrelationships Section). Hence, there is a vicious cycle between protein and riboflavin metabolism. Protein deficiency results in increased excretion of riboflavin and, in turn, riboflavin deficiency interferes with protein metabolism (Foy and Mbaya, 1977). In keeping with these findings, prolonged ariboflavinosis causes decreases in the levels of DNA and RNA in liver (Chatterjee et al., 1969).

While the level of protein in tissues is unaltered by ribo-flavin deficiency (Mookerjea and Hawkins, 1960), the concentration of the flavin coenzymes and the activities of enzymes that require FMN and FAD are depressed in most tissues and in the blood (Burch et al., 1956; Goldsmith, 1975). Some flavin-requiring enzymes are more sensitive to depletion than others and appear to parallel the reduction in levels of FMN and FAD. In erythrocytes riboflavin deficiency reduces both EGR and methemoglobin reductase (Beutler, 1969). In contrast, liver GR activity is only marginally affected by ariboflavinosis (Bamji and Sharada, 1972). Many non-flavin enzymes are unchanged or actually increased in activity as a conse-quence of riboflavin deficiency (Mookerjea and Jamdar, 1962; Chatterjee and Ghosh, 1967).

Riboflavin levels in liver diminish at a faster rate than that in red cells during progressive ariboflavinosis (Bamji and Sharada, 1972) while kidney flavin levels remain high (Glatzle et al., 1973). Concentrations of FMN are decreased proportionately more than concentrations of FAD. This difference in flavin levels

is likely due to the decreased flavokinase activity and increased
FAD pyrophosphorylase activity that occurs in riboflavin deficiency
(Rivlin et al., 1968; Rivlin, 1970b). Since FAD is required by
more enzymes than is FMN, Rivlin (1970b) has proposed that the
body conserves FAD at the expense of FMN when riboflavin is
limiting.

Hematologic Dyscrasia. Anemia is another consequence of ribo-
flavin deficiency. Most data have shown that ariboflavinosis is
associated with normocytic, normochromic anemia with decreased
erythroid formation in the bone marrow (Foy et al., 1961; Lane
et al., 1964). Since there is no alteration in red blood cell size
or white blood cell morphology, it is unlikely that deranged folic
acid or vitamin B_{12} metabolism contribute to this hematologic
defect (Lane et al., 1964, 1965).

Increased red cell fragility has been noted in vitro, but
there is no evidence of a shortened red cell life span in vivo
(Hassan and Thurnham, 1977). The anemia due to riboflavin de-
ficiency is characterized by a selective hypoplasia of the ery-
throid series of blood cells (Foy et al., 1961; Lane et al., 1964;
Lane and Alfrey, 1965; Foy et al., 1968, 1972; Lane et al., 1975).
In this disorder there is a decreased incorporation of iron into
erythrocytes which is normalized by riboflavin therapy accompanied
by remission of the aplasia.

Interestingly, the same defects have also been found in
African children with protein-calorie malnutrition (PCM). These
hematologic abnormalities respond either to corticosteroid or
riboflavin therapy (Kondi et al., 1967; Foy and Kondi, 1968).
Examination of the adrenal glands in both riboflavin-deficient
baboons and children with PCM reveal similar pathological findings
regarding the presence of focal hemorrhages, fibrosis and cellular
atrophy in various zones (Foy and Kondi, 1968). It appears that
ineffective erythropoiesis in riboflavin deficiency and PCM are
related to defective steroid metabolism. Further study has demon-
strated that corticosteroid levels in blood and urine are lower in
riboflavin deficiency and do not respond normally to ACTH stimula-
tion. All of these findings point to the possibility that ribo-
flavin deficiency causes erythroid hypoplasia by affecting the
production of corticosteroids which in turn, are responsible for
the stimulation of erythropoiesis of the bone marrow.

Neurological Abnormalities. There is some evidence that ribo-
flavin status is important both in peripheral and central nervous
system functions. Observations concerning burning feet syndrome
suggest that riboflavin deficiency may be a factor in causing
diminished nerve conduction as evidenced by reduced sensory per-
ception of temperature, touch and vibration (Lai and Ransome,
1970; Lane et al., 1975) as well as decrement of the hand-grip

strength (Sterner and Price, 1973). Microscopic and ultrastructural studies in experimental animals have shown a degeneration of the myelin sheath of peripheral nerves (Shaw and Phillips, 1941; Street et al., 1941; Norton et al., 1976) and the posterior columns of the spinal cord (Street et al., 1941); cellular organelles both of the myelinated and non-myelinated nerve fibers remained intact. These abnormalities are possibly due to the deranged β-oxidase of fatty acids that occurs during riboflavin deficiency; if such is the case, these abnormalities are not caused by reduced coenzyme B_{12} status (Frenkel et al., 1979).

Inadequate riboflavin nutriture also appears to alter central nervous system functions by causing defects in the cellular metabolism of the brain (Nutr. Rev., 1975). These defects manifest themselves as behavioral and/or electrophysiological abnormalities (Brozek and Vaes, 1961; Arakawa et al., 1968a; Sterner and Price, 1973). If riboflavin deficiency occurs during the critical period of cellular proliferation in young growing animals, there is a decrease in the DNA content of brain (Fordyce and Driskell, 1975) which is not corrected by repletion during or after weaning. Other studies indicate that riboflavin-deficient rats score lower in tests of learning ability and that this impairmant is even more pronounced if the ariboflavinosis transpires in utero (Muenzinger et al., 1937).

Congential Malformation. A popular dictum is that malnutrition in general and ariboflavinosis in particular can complicate pregnancy and greatly compromise fetal outcome (Balfour, 1944; Chaudhuri, 1971; Heller et al., 1974; Smithells et al., 1976). Extensive studies (Warkany and Nelson, 1942; Warkany and Schraffenberger, 1943, 1944; Warkany, 1975) have shown that riboflavin deficiency in rats provokes widespread metabolic disturbances and teratogenesis. Severe riboflavin deficiency may prevent conception while moderate deficiency can cause prematurity, congenital abnormalities, fetal resorption and stillbirths. A slight depletion of riboflavin will not necessarily have any deleterious effect on the embryo. It is not known if these effects are caused by the reduced electron transport system activity that occurs in riboflavin-deficient rats during the critical period of organogenesis (Aksu et al., 1968).

A similar relationship between maternal riboflavin deficiency and fetal malformation has not so far been recognized in man (Brzezinski et al., 1952; McGanity et al., 1969; Heller et al., 1974; Rosenthal, 1978). Smithells et al. (1976) have reported the occurrence of neural tube defects (anenecephaly, meningocele, hydrocephalus or microcephalus) in six infants who were born to mothers that had had low blood riboflavin, folacin and vitamin C levels during the first trimester of pregnancy. The role of pure riboflavin deficiency in causing the neural tube defects remains

to be clarified.

Carcinogenesis and Mutagenesis. Riboflavin deficiency has
been suggested to be both a preventive and causative factor in
carcinogenesis (Rivlin, 1975; Foy and Mbaya, 1977). Urinary
excretion of riboflavin tends to be lower in cancer patients
(Bamji and Ahmed, 1978) and may reflect poor dietary intake and/
or increased utilization by the tumor cells. Depriving rapidly
growing tumors of flavin coenzymes vital for metabolism by in-
tentionally producing riboflavin deficiency may prove useful in
the treatment of certain malignancies such as polycythemia vera
and lymphoma (Lane, 1971). In contrast, riboflavin deficiency in
baboons can produce cellular metaplasia which is strikingly similar
to the lesions of primary esophageal cancer in man (Foy and Mbaya,
1977).

Riboflavin may also play a role in mutagenesis. Recall that
phototherapy of hyperbilirubinemia in infants can cause riboflavin
deficiency (Causes of Deficiency Section) except in those neonates
with G-6-P-D deficiency (Lopez et al., 1978). This finding,
coupled with the observation that riboflavin potentiates the effect
of blue light phototherapy by enhancing the rate of bilirubin de-
composition (Kostenbauder and Sanvordeker, 1973; Sanvordeker and
Kostenbauder, 1974; Pascale et al., 1976), provides rather sub-
stantial rationale for riboflavin supplementation. However, the
addition of a physiological dose or riboflavin to either calf
thymus DNA or cultured He La cells which are subsequently irradi-
ated by blue light of the same wavelengths used in phototherapy
results in alterations of the physiological properties of the DNA
(Speck et al., 1975).

Immunology. Animal studies have shown that riboflavin de-
ficiency is accompanied by a reduction in the levels of IgM and
IgG and the number of antibody-forming cells of the spleen
(Pruzansky and Axelrod, 1955; Kumar and Axelrod, 1978). The clin-
ical significance of these observations during periods of in-
fectious illnesses and convalescence with concomitant aribofla-
vinosis is not clear (Beisel et al., 1972; Feigin, 1977). It seems
unlikely that a relatively brief febrile illness in a well-nourished
adult will produce a long-term derangement in vitamin metabolism
(Feigin, 1977) and require vitamin therapy. Chronic illness, how-
ever, is likely to deplete body stores of riboflavin. More know-
ledge about the role of riboflavin deficiency in altering cell
mediated immunity is required to understand the relationship of
ariboflavinosis to infection and even tumor immunology.

Drug Interactions. Certain drugs are known to interfere with
riboflavin metabolism. Examples include phenothiazine (Pinto
et al., 1979), oral contraceptive agents (Briggs and Briggs, 1974;
Sanpitak and Chayutimonkul, 1974), diphenylhydantoin (Saad and

Shehata, 1978), and boric acid (Roe, 1971; Roe et al., 1972).
Phenothiazine, a major tranquilizer, inhibits the conversion of
riboflavin to FAD by virtue of its structural similarity to ribo-
flavin. This drug causes increased urinary riboflavin excretion
and alters overall riboflavin status resulting in an increased
EGR AC. Since phenothiazine is widely applied in the treatment of
psychotic patients, it is important that more information about its
interaction with riboflavin be obtained.

     The effect of oral contraceptive agents (OCA) on riboflavin
status is controversial (Briggs and Briggs, 1974; Sanpitak and
Chayutimonkul, 1974; Ahmed and Bamji, 1976b; Guggenheim and Segal,
1977; Briggs, 1979; Carrigan et al., 1979). This discrepancy
may relate to the women's nutritional status prior to OCA usage.
Decreased blood levels in women taking OCA is apparently not the
result of diminished absorption (Carrigan et al., 1979) but may be
due to a preferential redistribution of riboflavin to the liver
where it is incorporated into flavoproteins (Ahmed and Bamji,
1976a).

     Diphenylhydantoin can cause reduced hepatic riboflavin stores
(Saad and Shehata, 1978) and ingestion of boric acid increases
urinary riboflavin excretion. Finally, alcohol ingestion can cause
riboflavin depletion. In none of these examples is the mechanism
known with certainty. It is likely that alcohol interferes with
riboflavin metabolism in several ways (see Causes of Deficiency
Section).

     Dietary riboflavin deficiency is known to diminish liver drug
metabolizing capability (Zannoni and Sato, 1976). Thus persons
subsisting on suboptimal amounts of dietary riboflavin, and at the
same time are using one of the above drugs, can be expected to be at
increased risk of developing ariboflavinosis.

TREATMENT

     When the presence of riboflavin deficiency has been diagnosed,
treatment by oral administration of 5 to 10 times the daily RDA
for riboflavin usually gives satisfactory results. Larger doses
can be administered with impunity but may not greatly reduce re-
covery time. In those instances where absorption is impaired,
riboflavin may be given intramuscularly or diluted in intravenous
fluid.

     The common practice among clinicians to recommend taking 5 mg
of the vitamin two or three times a day is really not warranted.
Regarding therapeutic regimens, multiple doses of riboflavin are
no more effective than a single dose (Garber et al., 1949; Chapman
and Campbell, 1955; Morrison and Campbell, 1960a). Divided doses
produce sustained urinary riboflavin excretion but have no effect

on the total amount excreted, i.e., dose size has no effect on the
percent of riboflavin absorbed by the body (Melnick et al., 1945;
Morrison et al., 1959).

There is considerable evidence that the pharmaceutical form
of riboflavin has a marked influence on vitamin availability.
Sugar-coated tablets which do not disintegrate in the body within
60 minutes are not completely available for absorption (Chapman
et al., 1954; Morrison et al., 1959). Enteric-coated tablets
behave similarly (Morrison and Campbell, 1960b). Even the light
coatings used on chewable preparations can reduce riboflavin avail-
ability by 50% (Morrison and Campbell, 1962). Despite advertising
claims, time-release vitamin formulations do not maintain blood
levels of riboflavin because most of the vitamin has moved past its
site of intestinal absorption within ninety minutes from the time
of ingestion (Morrison et al., 1960).

Without treatment the clinical lesions of ariboflavinosis
heal poorly (Horwitt et al., 1949). Improvement of the clinical
lesions is generally seen within a few days of therapy followed by
complete healing within 10 days to a few weeks. Biochemical abnor-
malities have been found to improve within 72 hours to a few days
(Greene, 1972; Rosenthal et al., 1973). Actual recovery time is
dependent upon the extent of depletion of total body stores and
the absence or presence of precipitating causes. Maintenance
dosage equal to the RDA requirement should be given to assure opti-
mal intake for a while after the lesions have healed. As men-
tioned previously, since pure riboflavin deficiency is rarely
observed in clinical practice, other nutrients should often be
prescribed concomitantly.

The increased riboflavin requirements of the pregnant woman
can generally be met simply by the consumption of additional
quantities of food to satisfy her extra caloric needs (Committee
on Maternal Nutrition, 1970). Opinions regarding the value of any
further and specific riboflavin supplementation during pregnancy
are divided. On the one hand, a significant percentage of pregnant
women have subclinical riboflavin deficiency (Heller et al., 1974)
and supplementation of deficient diets has improved the clinical
outcome of pregnancy (Brzezinski et al., 1952). On the other hand,
Kaminetzky et al. (1973) did not find that vitamin supplementation
influenced the incidence of riboflavin deficiency and found that
mothers with clinical symptoms of ariboflavinosis can have normal
pregnancies and deliveries with no deleterious effects on their
infants (Brzezinski et al., 1947). Another reason for not supple-
menting is that the crucial period of organogenesis is usually
over by the time that most women seek medical attention; any benefit
of remedial therapy is unlikely (Clarke, 1976). Riboflavin therapy
may be advisable when anemia in pregnancy is being treated (Clarke,
1973b, Ramachandran and Iyer, 1974).

The usefulness of providing riboflavin supplements to women
using OCA is equivocal. There is certainly no compelling evidence
to indicate that well-nourished women taking OCA develop riboflavin
deficiency. In malnourished women the administration of multi-
vitamins containing 2 mg of riboflavin daily or twice this dose
only during the 7 'non pill' days in the 28 day cycle, prevented
the OCA effect on riboflavin status but did not correct the initial
state of riboflavin deficiency that existed prior to the use of
OCA (Bamji et al., 1979). Higher doses of riboflavin may be re-
quired to achieve the latter.

The wisdom of riboflavin supplementation in cases of neonatal
hyperbilirubinemia being treated with phototherapy is questionable
at this time (see Pathophysiology Section). A final decision must
consider the potential risk of mutagenesis.

Treatment of disease is a clinican's primary goal but nutri-
tional support must always be an integral component in the practice
of good patient care. Perhaps the most significant attribute of
good nutrition is the increased resistance to disease and the pro-
motion of optimal growth, repair and maintenance of good health.
Equally important are efforts to minimize and hopefully eliminate
ariboflavinosis. Prevention is always a better alternative than
treatment. Knowledge of food sources, food preparation, absorption,
transport, storage and function of riboflavin is indispensable as
a preventive measure in protecting against severe ariboflavinosis
or reappearance of chronic riboflavin depletion.

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ROLE OF VITAMIN C IN HEALTH AND DISEASE

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INTRODUCTION

The biosynthesis, physiological functions and catabolism of
L-ascorbic acid (Vitamin C) in man remain topics of great interest
and challenge for the clinical biochemist. L-ascorbic acid is a
water-soluble vitamin, which chemically is a lactone (internal
ester of a hydroxycarboxylic acid) and is characterized by the ene-
diol group which makes it a strongly reducing compound. Ascorbic
acid itself is the most active reducing agent known to occur natu-
rally in living tissues. The empirical formula of this vitamin
is $C_6H_8O_6$. The Levo-ascorbic acid is an active antiscorbutic
substance, while the dextro-ascorbic acid is not (Figure 1). One
of the isomers, D-isoascorbic acid or erythorbic acid, is produced
commercially for use as a food additive. It has the reducing
power of L-ascorbic acid but cannot support growth. L-ascorbic
acid is readily and reversibly oxidized to dehydro-L-ascorbic acid
which retains vitamin C activity (Figure 2). This compound can be
further oxidized to diketo-2-gluconic acid, in a non-reversible
reaction; the compound has no reported biological activity. De-
hydration and decarboxylation can lead to the formation of furfural,
which can polymerize to form brown pigments or combine with amino
acids in the Strecker degradation. The failure of man, non-human
primates, guinea pigs and a few other species of animals to synthe-
size their own vitamin C from glucose is due to a deficiency of an
enzyme in the liver which converts L-gluconic acid to ascorbic acid.
Thus, for these animals vitamin C is an essential vitamin that is
required in the daily diet. While scurvy is not as common today in
the U.S.A. as compared to a century ago, vitamin C deficiency is
one of the more common vitamin deficiencies, particularly in school-
age children (Ten State Nutrition Survey, 1972).
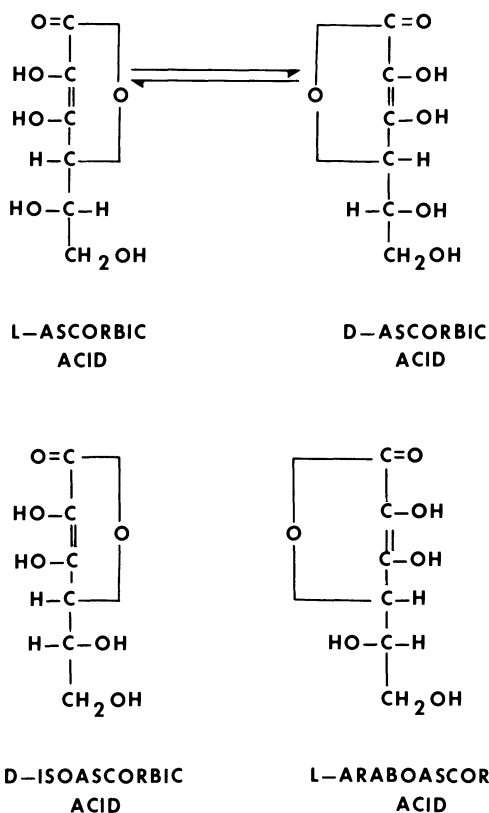
Fig. 1. Structural formulas of ascorbic acid and its stereoisomers.
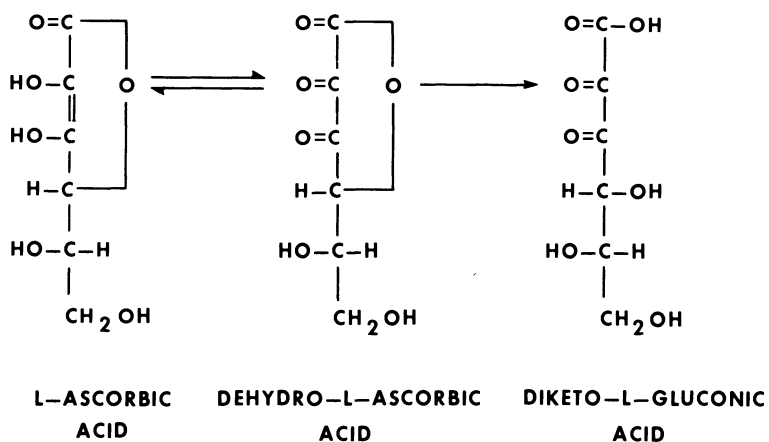
Fig. 2. Oxidation of L-ascorbic acid.

SYNTHESIS

     Vitamin C is a common and essential nutritional element of all
animals. Except for the invertebrates, fishes, Indian fruit-eating
bat, guinea pig, non-human primates and man, vitamin C can be syn-
thesized by plants and most animals (Figure 3). The emergence of
the biosynthetic ability of amphibians suggests that a greater need
of the vitamin was somehow linked with the evolution of vertebrates
from aquatic to the terrestrial environment. It is likely that the
evolution of all lines of vertebrates generally required loss of
some specialization as new specialization came about. It is dif-
ficult to determine why vitamin C is made in the kidney and not
the liver of amphibians. It may be considered that the early am-
phibians started synthesis in an organ where ascorbic acid could
be produced at a high rate. In vitro results show that in the
toads and frogs the activity of the kidney enzyme (L-glucono
oxidase) is much higher than that of the mammalian liver enzyme
(Chatterjee et al., 1975). The amphibians' capacity to synthesize

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                              ⎧         MAN
                              ⎪          ↑
                              ⎪   NON-HUMAN PRIMATES
              INCAPABLE  ⎨          ↑
                              ⎪      GUINEA PIG
                              ⎪          ↑
                              ⎩  INDIAN FRUIT-EATING BAT
                                          ↑
                              ⎧       MAMMALS
                              ⎪          ↑
              LIVER      ⎨
                              ⎪       AVIANS
                              ⎩     (Higher Order)
                                          ↑
                              ⎧       AVIANS
                              ⎪     (Lower Order)
                              ⎪          ↑
              KIDNEY     ⎨       REPTILES
                              ⎪          ↑
                              ⎩     AMPHIBIANS
                                          ↑
                              ⎧        FISHES
              INCAPABLE  ⎨          ↑
                              ⎩    INVERTEBRATES
```

Fig. 3. Schematic diagram of vitamin C - synthesizing abilities
         of various species of animals in relation to their phylo-
         geny (adapted from Chatterjee, 1973).

vitamin C in the kidney continued in that of the reptiles, but
was then transferred to the liver of mammals. This change in site
of synthesis appeared to have taken place when the vertebrates were
evolving thermoregulatory mechanisms and changed from poikilothermic
to homeothermic species. The kidney activities had been altered to
accommodate the necessities of life on dry land with increased
physiological demands such as regulation of calcium, phosphate,
urea and other analytes. Primitive birds retained the ascorbate
biosynthetic activity in the kidney, while the passeriform birds
(i.e., house crow and myna) can be considered transitional, on
the borderline in transferring the biosynthetic capacity from the
kidney to the liver. Thereafter, the ascorbate synthetic capacity
was taken over by the liver of the more evolved passeriform birds,
while a number of other highly evolved passerers are incapable of
producing vitamin C. The failure of the guinea pig, Indian fruit-
eating bat, non-human primates and man to synthesize ascorbic acid
is due to a common defect, namely, the absence of the terminal
enzyme L-glucono oxidase (Burns, 1957). Figure 4 illustrates the
pathway of ascorbic acid synthesis in animals via the glucuronic
acid pathway, the main substrate for biosynthesis being D-glucose.

CATABOLISM

    The metabolic fate of ascorbic acid and its derivatives in
animals depends on a number of factors including animal species,
route of ingestion, quantity of material, nutritional status, and,
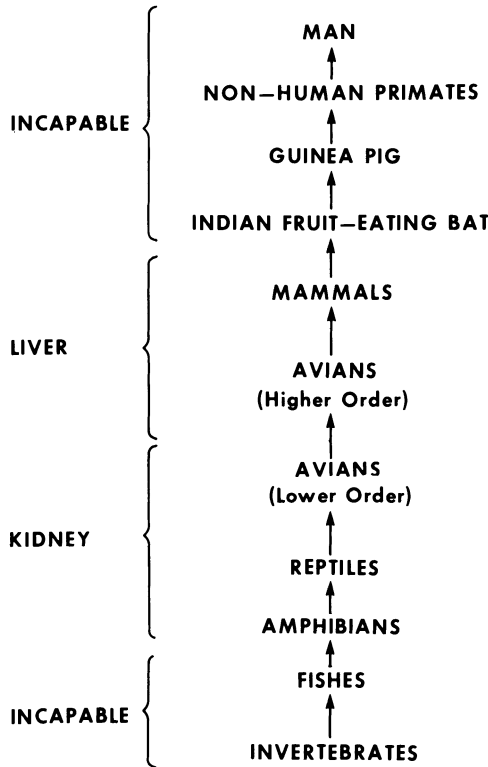of course, the compound itself. There appears to be a central
core of metabolism of ascorbic acid that is observed with low
dietary intakes and that is similar from species to species. Excess
ascorbic acid is handled quite differently in different species.
Thus, in man there is a limited intestinal absorption followed by
an efficient urinary excretion of the excess ascorbic acid. In
the guinea pig, some 60-70% of ingested ascorbic acid is rapidly
catabolized to CO_2. What, if any, part of the central core me-
tabolism of ascorbic acid is related to the nutritional requirement
for vitamin C is not known. The catabolism to CO_2 does not appear
to be a required function, since it does not occur in man (Figure 4).

    This metabolic path seems well defined in animals through
xylonate and lyxonate, but definite proof for the subsequent steps
and isolation studies on the enzyme involved are not available
(Tolbert et al., 1975). In particular, the question of whether the
catabolic pathway proceeds through xylitol or through the L-ribulose
to D-xyulose step for the conversion of L to D sugars is unresolved.
In this process ascorbate is first converted to dehydroascorbic acid
by a variety of enzymic and nonenzymic processes in animals. No
specific ascorbic acid oxidase has been reported in animals. Enzymic
delactonization of dehydro-L-ascorbic acid has been studied in animal
tissue (Kagawa et al., 1961; Kagawa and Takiguchi, 1962). The enzyme
is present in ox, rabbit, rat, and guinea pig livers, but is

Fig. 4. Pathway of ascorbic acid synthesis.

essentially absent in monkey and man, a result consistent with the absence of $^{14}CO_2$ production for ascorbic-1-^{14}C acid in man (Baker et al., 1966). 2,3-Diketogulonate decarboxylase has been studied in vivo and in vitro (Kagawa and Takaguchi, 1962) and the products shown to be L-xylonate and L-lyxonate. It is a widely distributed enzyme, probably present in all animals, including man.

Another pathway for the catabolism of ascorbate is by C(2)-C(3) carbon chain cleavage, to give oxalate and a 4-carbon intermediate. This pathway appears in all animals and represents about 4-10% of the essential catabolism of ascorbate in man. Banay and Dimant (1962) used double labeling to show that the 2 carbons of oxalate come from the C_1 and C_2 carbons of ascorbate. It is commonly suggested that oxalate is produced from 2,3-diketogulonate, because oxalate is formed in the spontaneous decomposition of ascorbic acid solutions.

The 4-carbon metabolites of ascorbic acid from C(2)-C(3) cleavage do not appear to be extensively catabolized in man, because very little of the label from ascorbate-4-^{3}H gets into the body water pool (Tolbert et al., 1967). In guinea pigs the body water pool is extensively labeled by ascorbate-4-^{3}H, probably by the catabolism of a 5-carbon compound formed by decarboxylation of 2,3-diketogulonate. Even so, threonate and threonolactone are also present in guinea pig urine, probably derived from the oxalate path for ascorbate catabolism, including that of man (Baker et al., 1971).

Ascorbate-3-sulfate has recently been shown to be a metabolite of ascorbic acid in urine of animals (Baker et al., 1971). The rapid clearance of ascorbate sulfate in blood is one of the possible reasons for the absence of antiscorbutic effect with ascorbate sulfate in the guinea pig. Ascorbate sulfate is ineffective in preventing or curing scurvy in guinea pigs whether given orally or by injection. A role of ascorbate sulfate as a sulfating agent has been demonstrated in biological systems (Bond, 1975), but further work in this area is needed.

PHYSIOLOGY

Collagen Metabolism

Direct involvement of ascorbic acid in collagen synthesis is well known and represents, perhaps, the most clearly defined biochemical role of this vitamin. The function of ascorbic acid in collagen synthesis has been reviewed by a number of investigators (Barnes and Kodicek, 1972; Barnes, 1975).

Studies in isolated collagen-synthesizing systems have demonstrated that ascorbic acid participates in the synthesis of collagen

hydroxyproline and hydroxylysine, both of which are formed by the
hydroxylation of particular prolyl and lysyl residues previously
incorporated into peptide linkage during the process of ribosomal
collagen protein synthesis. The precise mode of action of the
vitamin in these hydroxylations has yet to be elucidated.

The need for ascorbic acid as a reductant in vitro in these
reactions is not highly specific. Because of this lack of speci-
ficity, the possibility existed that compounds other than vitamin C
might perform this function in vivo and that impaired collagen
synthesis in scurvy might imply a role for ascorbic acid in collagen
metabolism other than one in hydroxylation. There appears to be
no evidence, however, for the participation of other reductants in
the hydroxylation mechanism in vivo. Studies in scorbutic guinea
pigs have indicated that hydroxylation at least of peptidyl proline
is impaired in vivo in ascorbic acid deficiency, and it is possible
that this is the primary lesion in collagen synthesis in scurvy.

Nevertheless, hydroxylation of collagen proline in vivo is only
slightly impaired in scorbutic animals. There is no evidence for
the occurrence of a substantially underhydroxylated moiety. It
is believed that a slight additional reduction beyond an initial
5-10%, which does not appear to impair collagen function, causes
a lack of formation of the triple helical structure of collagen
with consequent degradation of the unassociated α-chains. Accu-
mulation of the latter within the cell may also cause a feedback
inhibition of further collagen protein synthesis. The continuous
absence of accumulation of unhydroxylated material in the tissues
of scorbutic guinea pigs may be attributable to reduced collagen
protein synthesis, which is regarded as a secondary feature
of impaired hydroxylation.

Lipid Metabolism

In the last few years, there has been considerable interest
in vitamin C and its role in lipid metabolism and atherosclerosis.
In this regard vitamin C seems to be involved in at least two
systems: the maintenance of vascular wall integrity and the
metabolism of cholesterol to bile acids.

The etiology of atherogenesis has not been fully elucidated.
However, there appear to be several possible mechanisms for the
initial development and progression of the arterial vascular disease;
one possible common denominator being a change in vascular wall
permeability (Gore and Stefanovic, 1967). Since collagen is a major
component in the arterial blood vessel, it is not unreasonable
to suspect that a dysmetabolism of collagen may later alter the
arterial wall structure and ultimately affect vascular wall perme-
ability, predisposing the blood vessel to the development of
atherosclerotic lesion.

The guinea pig, like man, non-human primates, certain birds and the Indian fruit-eating bat, but unlike most other species, cannot synthesize vitamin C and is therefore dependent upon exogenous sources for meeting its daily requirements. It has been reported that there is an inverse relationship between liver ascorbic acid concentration and hepatic cholesterol concentration in guinea pigs (Ginter and Ondreicka, 1971; Ginter et al., 1967). Cholesterol accumulation in the liver during low ascorbate intake did not appear to originate from increased synthesis or absorption during times when liver ascorbate was low. However, decreased liver ascorbate appeared to decrease the conversion of cholesterol to bile acids for excretion via the bile (Ginter et al., 1971). Vitamin C may be a necessary cofactor for the hydroxylation of the cholesterol nucleus to form bile acids. It was concluded that this decreased rate of cholesterol catabolism and excretion leads to hypercholesterolemia.

There is a wide divergence of opinion concerning the significance of vitamin C levels in relation to atherosclerosis in human beings. Willis (1953) noted that there frequently was a deficiency of ascorbic acid in the arterial wall of hospitalized patients, and that old age seemed to accentuate the deficiency. He also noted that the lowest levels were in areas most prone to development of atherosclerosis (Willis and Fishman, 1955). Sokoloff et al. (1967) reported that of 20 atherosclerotic, hypercholesterolemic patients, the serum cholesterol decreased 30 percent and the β-lipoprotein decreased 25 percent after a 30-day course of 0.5 g of ascorbic acid three times a day. Normal subjects showed a significant decrease in serum cholesterol levels when 1.0 g ascorbic acid was added to theri diet, while those with atherosclerosis receiving clofibrate for treatment of hyperlipidemia or anticoagulant therapy tended to show increased cholesterol levels with high vitamin C intake (Spittle, 1972). Young, normal subjects, when given 1.0 g ascorbic acid per day for 6 weeks in addition to their usual dietary intake, failed to show any significant change in serum cholesterol level (Anderson et al., 1972). Ginter et al. (1970) found decreased plasma cholesterol levels after ascorbic acid therapy in hypercholesterolemic subjects (>200 mg/dl) with initial low blood ascorbic acid concentrations. In another study Ginter (1975) reported that 1 g of ascorbic acid daily for a period of 3 months given to subjects 50-75 years of age with a mean serum cholesterol concentration of <200 mg/dl had no effect on plasma cholesterol. Peterson et al. (1975) administered 4 g of ascorbic acid daily for 2 months to hypercholesterolemic patients and found no effect on serum lipids.

Since there are conflicting reports on the effects of ascorbate on serum cholesterol levels, we examined the relation of varying intake of ascorbate on serum lipid levels in the guinea pig and in human beings.

First of all, it should be recognized that some of the con-
fusion in the literature appears to be related to the fact that
several studies focused upon acute avitaminosis C rather than
chronic hypovitaminosis C. Furthermore, the studies used different
means of ascorbate administration (via drinking water, gastric
intubation, or intraperitoneal injection). Another factor that
may have influenced the results is the vehicle that was used in
the vitamin C solution. Some investigators used ascorbate in water
while others used sucrose to increase the palatability of the
ascorbic acid. In the present study, a commercial source was
used (Ce-Vi Sol from Mead Johnson and Co., Evansville, IN), in
which ascorbic acid was diluted in a glycerine solution to minimize
the oxidation of L-ascorbate to dehydroascorbate, a reaction that
occurs extremely rapidly. It is possible that those who provided
ascorbate in the drinking water did not obtain the effect of
ascorbate, but rather that of dehydroascorbate. Furthermore, when
vitamin C is provided in drinking water bottles, it is not possible
to predict the exact amount of ascorbate intake, because there
is much spillage when the guinea pigs drink. There is also the
problem of varying water intake by different animals.

In the first experiment, an acute vitamin C deficiency study
was made by feeding 10 adult male guinea pigs (English short-hair
strain) a chow diet (Reid-Briggs guinea pig diet, ICN Pharmaceu-
tical Inc., Cleveland, OH) completely devoid of vitamin C (Table 1).

Table 1. Composition of the Reid Briggs Guinea Pig Diet[a,b]

Nutrients	Percent (By Weight)
Vitamin-free casein	30.0
Starch, corn	20.0
Alphacel	15.0
Sucrose	10.3
Glucose	7.8
Corn Oil	7.3
Salt Mixture	6.0
Potassium acetate	2.5
Vitamin fortification mixture[c]	0.6
Magnesium oxide	0.5

[a] Reid and Briggs (1953).
[b] Semi-synthetic diet was made by ICN Pharmaceutical Inc.,
    Cleveland, OH.
[c] Vitamin C fortification normally consisting of 0.2 percent
    (by weight) was omitted when the scorbutogenic diet was
    made.

Ten age-matched control male guinea pigs fed a guinea pig chow
containing vitamin C were used to obtain baseline values. Ascor-
bate content in tissues and sera was measured by the method des-
cribed by Roe and Kuthner (1943). All lipid analyses were done
on fasting samples. A detailed description of the methods is
described elsewhere (Naito and Gerrity, 1979). The results are
presented in Table 2. After 2 weeks on the experimental diet,

Table 2. Effects of Acute Avitaminosis C ($\overline{X} \pm$ S.D.)[a]

Analytes	Initial Day 0[b] (Baseline Values)	Final Day 14[b]	Signifi-cance
Total weight change (grams)	925 + 68	-47.3 + 33.0	P<0.05
Serum Ascorbate (mg/dl)	0.64 + 0.41	0.052 + 0.04	P<0.01
Urine Ascorbate (mg/dl)	1.37 + 0.79	0.86 + 0.55	N.S.
Liver Ascorbate (mg/100 g wet wt.)	12.37 + 6.11	1.15 + 0.38	P<0.01
Adrenal Ascorbate (mg/100 g wet wt.)	86.77 + 29.22	8.05 + 2.45	P<0.01
Spleen Ascorbate (mg/100 g wet wt.)	36.11 + 11.8	4.17 + 0.6	P<0.01
Serum Cholesterol (mg/dl)	44.7 + 13.3	58.0 + 4.4	N.S.
Liver Cholesterol (mg/100 wet wt.)	302.6 + 62.8	328.8 + 26.5	N.S.
Serum Triglycerides (mg/dl)	61.8 + 26.7	146.6 + 50.3	P<0.1

[a] Animals were fed the Reid-Briggs guinea pig scorbutogenic
diet for 14 days.
[b] N=10 animals per group.
[c] Students t-test

signs of acute scurvy were demonstrated by microhemorrhages of
the small blood vessels of the tibia-femur joint. After 2 weeks
on the scorbutogenic diet, there was a significant reduction in
body weight. The level of vitamin C in most organs reached scor-
butogenic levels. However, the acute avitaminosis C condition
did not appear to be associated with changes in serum lipid levels.
Because the animals demonstrated signs of metabolic distress
(i.e., stopped eating, loss of body weight, and lethargy), it

appears that these types of acute studies may yield misleading
lipid results. This was confirmed after examination of data
that was collected by repeating the experiment; it was found
that the avitaminosis C condition resulted in a 21% elevation
of the serum cholesterol concentration rather than no change as
observed in the first study (unpublished report).

The following study was designed to examine the chronic ef-
fects of varying levels of vitamin C (Ce-Vi Sol) intake on serum
and liver cholesterol concentration in adult male guinea pigs fed
either a chow diet or a 2.0% cholesterol (by weight) supplemented
chow diet. The experimental design is presented in Table 3.

Table 3. Experimental Design -- Effects of Varying Levels
          of Vitamin C Intake With and Without Cholesterol
          Supplementation[a,b]

Ascorbate Intake mg/animal/day	Group	Diet
0.5	Hypovitaminosis C	Vitamin C Free, no cholesterol
0.5	Hypovitaminosis C	Vitamin C Free, cholesterol[c]
5.0	"Experimental Control"	Vitamin C Free, no cholesterol
5.0	"Experimental Control"	Vitamin C Free cholesterol
50.0	Hypervitaminosis C	Vitamin C Free, no cholesterol
50.0	Hypervitaminosis C	Vitamin C Free, cholesterol
Normal	Control	Chow

[a]Adult Male Guinea Pigs (N=6 animals/group).
[b]Animals were fed the Reid-Briggs guinea pig chow diet with
0.2 g% (w/w) vitamin C fortification.
[c]cholesterol = 2.0% (by weight) supplementation.

Since it has been reported that 5-10 mg/animal/day is the recom-
mended requirement for vitamin C for adult guinea pigs (Banerjee
and Singh, 1958; Ginter and Ondreicka, 1971), that daily dose
was arbitrarily adopted for the present studies. They are referred
to here as "experimental" controls. The 0.5 mg/animal/day group,
then, was considered hypovitaminosis C group, and the 50

mg/animal/day was considered hypervitaminosis C group. A fourth
group (control) was fed the standard guinea pig diet (Reid-Briggs),
which contained about 0.2 g percent of vitamin C (by weight). The
First three groups were fed the Reid-Briggs scorbutogenic diet
(Table 3) and were given daily vitamin C via gastric intubation
for 6 weeks, after which time the study was terminated. After
removing 2.5 ml of blood (for lipid studies) via the jugular vein,
an equal amount of Evans blue dye was infused and allowed to
circulate for 2 hours while the animals were under amytol anes-
thesia. The animals were then sacrificed and the tissues removed,
frozen with liquid nitrogen, and stored until analyzed. The aorta
was quickly removed, rinsed in buffered 1.3% formaldehyde solution,
and cut longitudinally, and the intima was examined for Evans
blue uptake.

     The effects of different levels of vitamin C intake on body
weight, serum and liver ascorbate, and cholesterol concentrations
of guinea pigs fed a low-cholesterol diet are shown in Tables 4
and 5. All animals gained body weight except the hypovitaminosis
C group, which lost weight. The liver ascorbate concentration
correlated positively with the increasing dietary intake of
vitamin C, while the serum ascorbate levels did not. It is inter-
esting that the 50 mg vitamin C group had a hepatic ascorbate
concentration similar to that of the control group. Through feed-
intake studies, it was estimated that 40-50 mg of vitamin C was
consumed per day by the control animals on the Reid-Briggs chow
diet containing 0.2% vitamin C (by weight). The presumed euvita-
minosis C group, 5 mg/day, had a significantly lower liver vita-
min C content than the control group. The serum cholesterol level
was lowest in the 50 mg/day group and highest in the 0.5 mg/day
group, suggesting an inverse relationship bewteen vitamin C intake
and serum cholesterol level. The hepatic cholesterol was higher
in all three experimental groups when compared to the control
group, but the 0.5 mg/day group had the most accumulation of
hepatic cholesterol.

     In another study, the Reid-Briggs chow diet supplemented with
2% cholesterol (by weight) was fed to three groups of guinea pigs
provided with different levels of vitamin C for 6 weeks. The
control group from the previous study was also used for this study.
The 50 mg/dl group maintained body weight, whereas the 5.0 and
0.5 mg/day groups lost weight (Table 5). It appears that with
a high cholesterol diet, the consumption of larger amounts of
vitamin C may be necessary to prevent the loss of body weight.
Like the group fed the low-cholesterol diet, those groups of
guinea pigs fed the high-cholesterol diet and given varying daily
amounts of vitamin C via stomach intubation showed a positive
correlation with the intake and hepatic ascorbate levels, but not
with the serum ascorbate concentration (Table 5). Thus, it
appears that while the serum ascorbate level is not a good index

Table 4. Effect of Different Levels Of Vitamin C Intake on Serum and Liver Lipid Concentration of Guinea Pigs Fed Chow Diet ($\bar{X} \pm$ S.E.M.)

Ascorbate Intake (mg/animal/day)[a]	Body Weight (g)	Ascorbate Serum (mg/dl)	Ascorbate Liver (mg/100 g tissue)[c]	Serum Lipids (mg/dl) Chol[d]	TG[e]	PL[f]	Liver Lipids (mg/100 g tissue)[e] Chol	TG	PL
Normal[b]	661 ±33.2	0.35 ±0.02	9.18 ±1.0	44.8 ±5.0	77.2 ±19.1	68.8 ±6.6	275.3 ±4.0	4227.9 ±591.4	3824.2 ±101.6
0.5	498 ±36	0.028 ±0.006	1.001 ±0.187	110.7 ±13.2	168 ±58.1	88.0 ±14.3	570.1 ±95.3	1887.3 ±444.4	3710.2 ±130.5
5.0	590 ±44	1.068 ±0.01	5.467 ±0.176	73.8 ±15.7	87.3 ±14.9	22.8 ±10.4	366.7 ±39.6	3133.2 ±1175.4	3063.2 ±54.6
50.0	535 ±27	0.1475 ±0.04	8.649 ±1.720	61.0 ±3.5	102.0 ±35.0	47.6 ±1.7	372.7 ±31.7	1426.5 ±334.6	3738.4 ±153.4

[a] Animals were fed Reid-Briggs scorbutogenic diet and given Vitamin C via tubal intubation.
[b] Animals were fed Reid-Briggs guinea pig chow diet with 0.2 g% (by weight) Vitamin C fortification.
[c] Wet weight
[d] Chol = Total cholesterol
[e] TG = Triglycerides
[f] PL = Total phospholipids

Table 5. Effect of Different Levels of Ascorbate Intake on Serum and Liver Lipids of Guinea Pigs Fed a Scorbutogenic Diet with 2.0 Percent Cholesterol-Supplementation ($\overline{X} \pm$ S.E.M.)

Ascorbate Intake (mg/animal per day)[a]	Final Body Weight (g)	Δ in Body Weight (g)	Ascorbate		Serum Cholesterol (mg/dl)	Liver Cholesterol (mg/100 g tissue)[c]
			Serum (mg/dl)	Liver (mg/100 g tissue)[c]		
0.5	381 ±24	(−96)	0.052 ±0.016	1.232 ±0.419	463.8 ±57.1	4803.5 ±326.2
5.0	414 ±5	(−48)	0.045 ±0.369	4.389 ±1.041	322.7 ±40.8	4539.9 ±450.5
50.0	544 ±28	(−7)	1.392 ±0.078	8.774 ±1.391	229.3 ±29.7	3371.7 ±736.9
Control[b]	661 ±33	(+183)	0.350 ±0.020	9.180 ±1.000	44.8 ±5.0	275.3 ±4.0

[a] Animals were fed Reid–Briggs scorbutogenic diet with 2.0 percent cholesterol supplementation and given vitamin C via tubal intubation.
[b] Animals were fed Reid–Briggs diet with 0.2 g percent (by weight) vitamin C fortification and no cholesterol supplementation. Calculated intake = 40–50 mg/animal/day.
[c] Wet weight.

for vitamin C intake, hepatic ascorbate levels are. Again the
50 mg/day group had a hepatic vitamin C content comparable to
that of the controls. Feeding the 2% cholesterol diet resulted
in an increase in serum cholesterol levels in all three experimen-
tal groups as compared to animals on a chow diet alone (Table 5).
The data suggested that an inverse relationship existed between
vitamin C intake and serum cholesterol level. However, even the
intake of 50 mg/day was not sufficient to normalize the serum
cholesterol levels. Similarly, the high vitamin C intake had little
effect on the accumulation of hepatic cholesterol. The Evans blue
dye study indicated that the guinea pigs fed the no-cholesterol
diet showed no evidence of dye uptake. However, about 30% of
the animals in the 0.5 and 5.0 mg ascorbate per day groups fed
2% cholesterol showed evidence of Evans blue uptake, as exhibited
by areas of streaking in the thoracic aorta. The 50 mg/day group
and the control animals did not demonstrate any Evans blue uptake
by the intima. It can be concluded from this study that:

    a.   Liver ascorbate level correlated with the amount of oral
        intake of vitamin C, while the serum ascorbate level
        did not.
    b.   Five mg vitamin C per animal per day was not an adequate
        amount for maintaining normal health and body weight in
        this strain of guinea pigs; 50 mg per animal per day may
        have represented a more adequate intake.
    c.   Cholesterol feeding caused a loss of body weight, par-
        ticularly in the animals with insufficient vitamin C
        intake.
    d.   Vitamin C insufficiency caused an increase in serum
        and hepatic cholesterol concentrations over a 6-week
        period in both cholesterol-fed guinea pigs and those
        fed no cholesterol.
    e.   About 1/3 of the animals in the 0.5 and 5.0 mg ascorbate
        per day groups fed the cholesterol diet showed Evans
        blue uptake in the thoracic aorta. The guinea pigs
        receiving no cholesterol showed no indication of such
        changes. The 50 mg ascorbate per day group fed choles-
        terol also showed no uptake of Evans blue.
    f.   This study suggested that vitamin C, in some way, does
        influence cholesterol metabolism in guinea pigs. Further
        studies are necessary to precisely define the role of
        vitamin C in cholesterol metabolism and arterial wall
        integrity.

It appears that a bolus dose of 50 mg vitamin C via stomach
intubation provided a sufficient amount of ascorbate to maintain
hepatic vitamin C levels comparable to those of control animals
who had vitamin C fortified in their diet. By bolus dose, it is
difficult to determine how much vitamin C was actually absorbed
by the gastrointestinal tract. The level of 8-9 mg ascorbate

per 100 g liver probably represents the saturated level. The question arises whether two 25 mg doses or five 10 mg doses per day lead to greater coefficient of absorption of vitamin C, thus leading to more effective action of ascorbate on cholesterol metabolism.

Since the 50 mg/day dose was neither effective in inhibiting the rise of nor lowering the serum cholesterol levels in the cholesterol-fed pigs, it would be interesting to determine whether the pharmacological or mega dosages, i.e., 250 or 500 mg/day vitamin C, can be effective. However, because our attempts to administer 250 mg/day to the animals were associated with diarrhea, it appears to be physiologically unpractical to experiment with higher dosages.

Results of our study with the guinea pigs suggest that insufficient vitamin C intake causes a rise in serum cholesterol concentrations. Since scurvy or hypovitaminosis C in man is rare today, it is difficult to extrapolate the usefulness of these animal studies to man. It is probably more important to know whether large dosages can act as hypocholesterolemic agents in man, especially when previous studies are in conflict (Willis and Fishman, 1955; Sokoloff et al., 1967; Spittle, 1972; Anderson

Time Period	Period 0			Period 1			Period 2			Period 3		
	1st Week	2-3 Weeks Later	4th Week (Time Zero)	End of 10th Week			End of 16th Week			End of 22nd Week		
Treatment	No Vitamin C or Placebo (Maintain Regular Diet)			On Vitamin C			Off Vitamin C (Placebo)			On Vitamin C		
				2nd	4th	6th	2nd	4th	6th	2nd	4th	6th
Physical exam			X			X			X			X
Dietary history	Briefing		X		X			X			X	
Laboratory tests (need blood and urine)												
Cholesterol	From chart	X	X	X	X	X	X	X	X	X	X	X
Triglyceride	From chart	X	X	X	X	X	X	X	X	X	X	X
Phospholipid		X	X	X	X	X	X	X	X	X	X	X
Lipoprotein electrophoresis	From chart	X	X	X	X	X	X	X	X	X	X	X
Total protein	From chart	X	X		X			X			X	
Protein electrophoresis	From chart	X	X		X			X			X	
SMA-12/60	From chart	X	X		X			X			X	
SMA-6/60 (kidney profile)	From chart	X	X		X			X			X	
Thyroxine	From chart	X	X		X			X			X	
Ascorbate	X	X	X	X	X	X	X	X	X	X	X	X

n = 6 subjects/group
Male only

Fig. 5.   Experimental design of vitamin C study on Type IIa hyperlipoproteinemic subjects: Group A.

Time Period	Period 0			Period 1			Period 2			Period 3		
	1st Week	2-3 Weeks Later	4th Week (Time Zero)	End of 10th Week			End of 16th Week			End of 22nd Week		
Treatment	No Vitamin C or Placebo (Maintain Regular Diet)			Off Vitamin C (Placebo)			On Vitamin C			Off Vitamin C (Placebo)		
				2nd	4th	6th	2nd	4th	6th	2nd	4th	6th
Physical exam			X			X			X			X
Dietary history	Briefing		X			X			X			X
Laboratory tests (blood)												
Cholesterol	From chart	X	X	X	X	X	X	X	X	X	X	X
Triglyceride	From chart	X	X	X	X	X	X	X	X	X	X	X
Phospholipid		X	X	X	X	X	X	X	X	X	X	X
Lipoprotein electrophoresis	From chart	X	X	X	X	X	X	X	X	X	X	X
Total protein	From chart	X	X			X			X			X
Protein electrophoresis	From chart	X	X			X			X			X
SMA-12/60	From chart	X	X			X			X			X
SMA-6/60 (kidney profile)	From chart		X			X			X			X
Thyroxine	From chart	X	X			X			X			X
Ascorbate		X	X	X	X	X	X	X	X	X	X	X

n = 5 subjects/group
Male only

Fig. 6. Experimental design of Vitamin C study on Type IIb hyper-
lipoproteinemic subjects: Group B

et al., 1972; Ginter et al., 1970; Ginter, 1975; Peterson et al.,
1975) as discussed earlier.

The following study was designed to determine whether one
gram (500 mg in the morning and 500 mg in the evening) of vitamin
C intake per day would lower serum lipids in persons with hyper-
β-lipoproteinemia (types IIa and IIb). The double blind, double-
crossover experimental design is shown in Figures 5 and 6. The
ten male participants (mean age = 45 years; all had cholesterol
concentration >300 mg/dl) were divided into two groups, one of which
(A) started with the vitamin C period first (two 250 mg tablets in
the morning and two 250 mg tablets in the evening), followed by the
placebo period (calcium lactate) and ending with the vitamin C
period. Group A consisted of type IIa hyperlipoproteinemic sub-
jects, while group B consisted of participants with type IIb hyper-
lipoproteinemia. The second group (B) started with the placebo
period first, followed by the vitamin C period and ending with the
placebo period. Each period but the baseline period (4 weeks)
lasted 6 weeks. A baseline period when blood samples were taken
about 2 weeks apart was set up at the initiation of the experiment.
During each of the three experimental periods, fasting blood
samples were collected at 2-week intervals. The mean levels

the parameters measured of the three bleedings for each period
were used to express the effect or lack of effect of vitamin C.
Since the participants were free-living, a dietary history was
recorded by a dietitian by 3-day detailed record keeping each week.
The composition of the dietary intake was calculated using tables
from Feeley et al. (1972), Watt and Merrell (1963) and Church
and Church (1977). The results are presented in Tables 6 and 7.
The dietary intake study indicated that the caloric intake, percent
of calories as carbohydrates, proteins, fat, alcohol, as well as
the ascorbate (besides the vitamin C tablets) and cholesterol
intake during each of the four periods (baseline periods 1, 2
and 3 were not statistically different from each other in both
groups A and B. The low cholesterol intake is due to the low-
cholesterol diet that the subjects were ingesting. The effect of
1 g of vitamin C per day on serum lipids is shown in Table 8.
Despite a 2.5 to 3-fold elevation in serum ascorbate levels during
the vitamin C periods (compared to baseline), the serum choles-
terol, triglyceride and phospholipid concentrations did not change
in either group A or B. To ensure that all the participants
consumed the placebo and vitamin C each day during the study, a
pill count was made every 2 weeks when they came in for bleeding
and physical examination. Compliance on the pills was 98% in group
A and 99% in group B during the entire study. Because high dosages
of vitamin C intake may result in oxylate stone formation in the
kidneys (Barness, 1975) and electrolyte imbalances (Lewin, 1974),
18 different analytes were monitored on the Technicon SMA 12/60
and 6/60 (1973). The results (Tables 9-12) indicated that little
change in the concentration of the various analytes occurred
during the different experimental periods. In addition, there
was no change in body weight, blood pressure, or serum thyroxine
concentration (in either group) during the different periods.
This study indicates that in adult male individuals with hyper-
β-lipoproteinemia, large intake of vitamin C does not appear to
be effective in lowering serum lipids. This does not negate
the possibility that during insufficient vitamin C intake derange-
ment of lipid and lipoprotein metabolism can occur.

Hematopoiesis

In addition to the above physiological functions, vitamin C
plays a number of roles that relate to hematopoiesis. Although
anemia is not a prominent component of clinical scurvy, it does
occur in some instances for a variety of reasons. Ascorbic acid
is a potent reducing agent and as such it enhances absorption of
iron and inhibits absorption of copper from the digestive tract
(Hodges, 1976). It also facilitates the transfer of iron from
transferrin to ferritin. Ascorbic acid aids in formation of
active compounds from tetrahydrofolates by linkage of the N^5 or
N^{10} position to formyl, hydroxymethyl, methyl or formimino groups
(Stokes et al., 1975). Massive doses of ascorbic acid, however,

Table 6. Group A: Daily Food-Intake Data During the Various Study Periods

Diet Components	Baseline Period	Ascorbic Acid Period	Placebo Period	Ascorbic Acid Period
Total Calories	1993.2 ± 389.81	2191.9 ± 408.16	2083.6 ± 49.04	2939.3 ± 787.81
% Calories from Carbohydrate	40.6 ± 5.69	42.3 ± 3.98	37.5 ± 1.84	37.6 ± 7.68
% Calories from Protein	20.0 ± 3.74	19.6 ± 2.33	22.0 ± 5.19	15.4 ± 3.44
% Calories from Fat	34.2 ± 7.15	31.8 ± 3.64	34.9 ± 8.63	35.4 ± 3.50
% Calories from Alcohol	5.2 ± 4.23	6.3 ± 3.29	5.6 ± 8.69	11.6 ± 8.10
Ascorbic Acid (mg)	138.2 ± 73.83	114.5 ± 30.29	86.5 ± 14.53	77.7 ± 27.08
Cholesterol (mg/dl)	328.4 ± 145.60	327.6 ± 164.55	366.4 ± 138.00	386.9 ± 89.27

Table 7. Group B: Daily Food – Intake Data During the Study Periods

Diet Components	Baseline Period	Ascorbic Acid Period	Placebo Period	Ascrobic Acid Period
Total Calories	1980.0 ± 392.11	1861.8 ± 277.37	2058.4 ± 267.74	1855.5 ± 277.48
% Calories from Carbohydrate	39.2 ± 15.52	35.0 ± 14.94	33.6 ± 11.30	34.5 ± 10.29
% Calories from Protein	19.9 ± 3.42	20.3 ± 3.55	18.9 ± 4.15	19.6 ± 3.81
% Calories from Fat	32.4 ± 7.74	32.2 ± 7.27	35.4 ± 5.15	35.0 ± 3.50
% Calories from Alcohol	9.5 ± 9.43	13.2 ± 14.35	12.8 ± 14.77	11.4 ± 11.38
Ascorbic Acid (mg)	120.0 ± 39.54	105.6 ± 20.33	130.0 ± 91.06	108.4 ± 46.52
Cholesterol (mg/dl)	293.5 ± 97.86	258.9 ± 79.72	294.6 ± 62.95	305.0 ± 63.44

Table 8. Serum Vitamin C and Lipid Levels of Group A and B Subjects (mg/dl, $\overline{X} \pm$ S.E.M.)

	Group A				Group B			
	Baseline	Period 1	Period 2	Period 3	Baseline	Period 1	Period 2	Period 3
TC	314.1 ± 10.8	309.9 ± 4.7	308.1 ± 5.9	299.9 ± 6.7	352.6 ± 13.0	353.9 ± 19.9	340.7 ± 15.9	338.3 ± 11.2
TG	93.4 ± 2.8	112.2 ± 5.3	103.1 ± 4.6	100.8 ± 3.6	211.6 ± 25.1	223.8 ± 25.9	212.1 ± 21.4	209.9 ± 23.3
PL	278.2 ± 9.3	271.1 ± 6.2	291.1 ± 10.1	273.8 ± 10.3	317.0 ± 10.1	303.5 ± 11.5	293.8 ± 11.7	313.5 ± 8.1
Serum Ascorbate	1.11 ± 0.14	2.05 ± 0.27	1.96 ± 0.12	1.96 ± 0.23	0.47 ± 0.14	1.80 ± 0.20	1.88 ± 0.35	1.83 ± 0.19

TC = Total Cholesterol
TG = Triglycerides
PL = Phospholipids
Group A = Type IIa hyperlipoproteinemic subjects
Group B = Type IIb hyperlipoproteinemic subjects

Table 9. Group A: SMA 12/60® Results of Various Serum Analytes of the Subjects During the Vitamin C Study ($\overline{X} \pm$ S.D.)

	T.P. (g/dl)	Alb. (g/dl)	Ca++ (mg/dl)	Inor Phos. (mg/dl)	Chol. (mg/dl)	Glu. (mg/dl)	Uric Acid (mg/dl)	T. Bili. (mg/dl)	Alk. Phos. (U/L)	CPK (U/L)	LDH (U/L)	SGOT (U/L)
Baseline	7.36 ±.07	4.54 ±.11	9.8 ±.2	3.30 ±.07	298 ±11	102 ±4	7.04 ±.26	0.78 ±.23	60 ±7	128 ±9	168 ±12	28.6 ±2.9
Period 1	7.34 ±.08	4.48 ±.05	9.5 ±.08	3.04 ±.19	301 ±39	96 ±5	6.7 ±.22	0.72 ±.22	58 ±7	112 ±10	162 ±8	3.0 ±2.7
Period 2	7.34 ±.03	4.42 ±.12	9.48 ±.19	3.16 ±.09	297 ±20	99 ±6	7.14 ±.22	0.84 ±.29	58 ±10	130 ±22	165 ±11	28.0 ±2.5
Period 3	7.4 ±.03	4.48 ±.09	9.58 ±.15	3.10 ±.18	297 ±16	101 ±4	6.66 ±.36	0.58 ±.09	57 ±8	121 ±12	164 ±10	27.5 ±3.2

Table 10. Group A: SMA 6/60® Results of Various Serum Analytes and of Thyroxine Levels of the Subjects During the Vitamin C Study ($\bar{X} \pm$ S.D.)

	Na^+ (mEq/L)	K^+ (mEq/L)	Cl^- (mEq/L)	CO_2 (mEq/L)	BUN (mg/dl)	Creat. (mg/dl)	T_4[a] (%)
Baseline	141 ±.5	4.56 ±.16	100 ±.8	28 ±1	20 ±3.2	1.24 ±.07	0.94 ±.02
Period 1	143 ±.5	4 ±.27	106 ±5	30 ±.9	21 ±3.3	1.35 ±.06	0.91 ±.01
Period 2	142 ±1	4.15 ±.17	101 ±2	28 ±.5	22 ±3.5	1.33 ±.1	0.92 ±.04
Period 3	142 ±.8	4.36 ±.1	102 ±1	29 ±.9	21 ±3.4	1.24 ±.04	0.93 ±.02

[a]As Free Thyroxine Index

Table 11. Group B: SMA 12/60® Results of Various Serum Analytes of the Subjects During the Vitamin C Study ($\overline{X} \pm$ S.D.)

	T.P. (g/dl)	Alb. (g/dl)	Ca++ (mg/dl)	Inor. Phos. (mg/dl)	Chol. (mg/dl)	Glu. (mg/dl)	Uric Acid (mg/dl)	T. Bili. (mg/dl)	Alk. Phos. (U/L)	CPK (U/L)	LDH (U/L)	SGOT (U/L)
Baseline	7.34 ±.17	4.55 ±.13	9.82 ±.11	3.46 ±.05	296 ±11	104 ±4	6.64 ±67	1.06 ±14	68 ±5	122 ±36	163 ±8	27 ±2.5
Period 1	7.18 ±.22	4.44 ±.81	9.5 ±.13	3.12 ±.14	304 ±5	108 ±6	6.88 ±.61	1.02 ±.24	69 ±3	145 ±24	166 ±12	26 ±3.3
Period 2	7.36 ±.11	4.48 ±.15	9.82 ±.25	3.28 ±.19	300 ±13	106 ±8	6.64 ±.62	1.12 ±.26	69 ±3	155 ±46	165 ±11	30 ±2.7
Period 3	7.26 ±.15	4.5 ±.13	9.58 ±.14	3.3 ±.09	296 ±15	106 ±7	6.96 ±.41	.84 ±.18	67 ±3	122 ±14	167 ±7	37 ±4.4

Table 12. Group B: SMA 6/60® Results of Various Serum Analytes and of Thyroxine Levels of the Subjects During the Vitamin C Study ($\bar{X} \pm$ S.D.)

	Na^+ (mEq/L)	K^+ (mEq/L)	Cl^- (mEq/L)	CO_2 (mEq/L)	BUN (mg/dl)	Creat. (mg/dl)	T_4[a] (%)
Baseline	142 ±.8	4.6 ±.09	103 ±.4	28 ±1.1	17 ±1.0	1.07 ±3.9	0.88 ±.02
Period 1	142 ±.6	4.68 ±.34	102 ±2	30 ±1.4	19 ±1.9	1.30 ±5.1	0.91 ±.02
Period 2	143 ±1	4.78 ±.47	103 ±1	29 ±.8	19 ±2.9	1.08 ±6.0	0.89 ±.02
Period 3	141 ±.7	4.58 ±.24	101 ±1	29 ±1.0	18 ±1.8	1.17 ±4.6	0.92 ±.03

[a] As Free Thyroxine Index

may have an adverse effect upon the availability of vitamin B_{12}
(Herbert and Jacob, 1974). Whether this proves to be of clinical
significance remains to be determined.

By inference if not by established fact, vitamin C is thought
to have a number of other functions related to hematology. Blood
clotting activity is obviously impaired in scurvy as manifested
by petechial hemorrhages and ecchymoses, bleeding gums and hemar-
throses. Despite claims that one or another of the blood clotting
factors may be impaired by lack of vitamin C other investigators
are unable to demonstrate any specific abnormalities of blood
clotting in scorbutic subjects although the patients may have
had obvious bleeding. It is true, however, that large amounts
of ascorbic acid can reverse the anticoagulant effects of either
heparin in vitro or dicumarol in vivo (Signell and Flessa, 1970).

One explanation for the bleeding that occurs in patients
with scurvy is lack of "intercellular substances" leading to
capillary leakage of blood. Biopsies of skin and subcutaneous
tissues from men with scurvy examined by electron-microscopy
failed to provide support for this theory (Hodges et al., 1969).
Furthermore Wolback's original article describing intercellular
substances probably related to collagen fibers that lie between
fibroblasts, not "intercellular cement", although he tended to
agree with previous workers who coined this term (Wolback and
Howe, 1926).

Sparing Effect on Other Vitamins

Ascorbic acid is known to have a sparing effect on several
other vitamins including those of the B complex group, vitamin A
and vitamin E. This may be related primarily to its role as an
antioxidant.

Common Cold

Vitamin C has been a popular medication for preventing the
common cold and reducing the severity of cold symptoms for many
years. Pauling's book on the subject has led to even further
use of vitamin C, in even larger doses (Pauling, 1970). Most re-
searchers today find that megadosages of vitamin C appear to have
little beneficial effect in terms of cold prevention in normal,
healthy individuals eating a balanced meal. Lewis et al. (1975),
for example, studied a population of 2,500 employees at the National
Institutes of Health and found that 3,000 mg of vitamin C intake
did not reduce the incidence of the common cold when compared
to the group receiving the placebo. This is in agreement with
the double-blind trial findings of Coulehan et al. (1974), Anderson
et al. (1972) and Wilson and Loh (1973) that vitamin C does not
have a prophylactic effect with doses ranging from 500-2,000 mg

per day. Likewise, vitamin C in 1.5 to 3 gram supplements has
failed to prevent colds induced by intranasal rhinovirus innocula-
tion (Sasaki et al., 1973; Schwartz et al., 1973). This does
not eliminate the possibility that inadequate intake of vitamin C
may make a person more susceptible to acquiring the common cold
or upper respiratory infections or syndromes. It should also be
pointed out that an adequate ascorbate intake for a normal, healthy
individual is not an adequate intake for chronically ill patients
or females taking oral contraceptive pills or during pregnancy.

The human requirement for vitamin C is increased during preg-
nancy. It appears to be increased in persons ingesting oral
contraceptive steroids. The rationale for increased requirements
is based primarily on the findings of a downward trend in plasma
ascorbic acid concentrations in successive trimesters of pregnancy
and decreased plasma, white cell, and platelet ascorbic acid
concentrations in women taking oral contraceptives (Rivers and
Devine, 1975).

The 1974 recommended dietary allowances (RDA) for vitamin C
is 60 mg/day for pregnant women and 80 mg/day for lactating women.
According to the data of Rivers and Devine (1975), this suggested
RDA during pregnancy and lactation may be markedly underestimated.

Stress

During the past decade vitamin C has become the most heavily
consumed vitamin, following revivals of the belief in its pro-
phylactic action on various diseases. In stress, vitamin C once
held a position of preeminence. Not only is ascorbate abundant
in the adrenal cortex, but its concentration fails promptly after
administration of adrenal corticotropic hormone (ACTH). It is
not known, however, if ascorbate is a necessary component of ste-
roidogenesis. On the contrary, accumulated evidence summarized
in man and experimental animals appears to militate against the
facilitating role of ascorbate in adrenal steroidogenesis (Kitabchi
and West, 1975). This is confusing in light of the fact that
vitamin C is found to be involved in the formation of norepinephrine
from dopamine and in the conversion of tryptophan to 5-hydroxyptophan
which is the first step in the synthesis of serotonin (Thoa and
Booker, 1963; Sulkin and Sulkin, 1967; Bhagat et al., 1966; Thoa
et al., 1966; Abboud et al., 1970; Cooper, 1961). Since two of
the important neurotransmitters are dependent upon ascorbic acid,
it is possible that vitamin C may have an important role in the
observed fatigue, weakness and vasomotor instability in scorbutic
patients. Fuller et al. (1971) have shown that vitamin C deficiency
in guinea pigs causes greater susceptibility to endotoxin shock.
This was expressed by a high mortality rate in scorbutic animals
within the first hour following sublethal dose of endotoxin admin-
istration. Morphologic changes in the animals which died from

the shock were most severe in the lungs and myocardium.

On the other hand, supplementation of vitamin C intake may
not necessarily aid a person in stress. Rats forced to swim in
ice water were reported to survive longer if they were given
supplements of ascorbic acid. Subsequent studies generally failed
to confirm the "anti-stress" actions of ascorbic acid in man.
Army troops transported to the top of a mountain where they were
subjected not only to high altitude hypoxia, but also to cold and
sleeplessness, nonetheless metabolized radioactively labeled
ascorbic acid at a normal rate. Other studies showed that athletic
performance did not improve following massive doses of vitamin C
(Ryer et al., 1954a, 1954b).

Prior to World War II, vitamin C in moderate doses was recom-
mended as a means to temporarily increase the "high-altitude re-
sistance" of pilots, airplane passengers and mountaineers (Petersen,
1941). However, Pfannenstiel (1938) and Doerholt (1938) also
reported that larger doses of vitamin C actually diminish the high-
altitude resistance. Thus, rabbits that had been given large amounts
of sodium ascorbate intravenously (100 mg each day for four days,
an additional 200 mg 30 minutes before the experiment) developed
convulsions typical of hypoxia with unprecedented violence in low-
pressure chambers under conditions that were well tolerated by
untreated animals. The same effect was in turn demonstrated with
human volunteers.

While it is fairly easy to make generalizations on the role
of vitamin C during stress, the dynamics of the body's metabolism
is too complicated to evaluate the effects on a singular basis.
First of all, the duration, frequency and type of stress are
independent components that result in varying effects of stress
on vitamin C requirements and metabolism. Secondly, the effects
of "stress" should be viewed as a systemic rather than a focal
effect. A case and point could be made with vitamin C supple-
mentation for stressed laying hens. The biochemical role of
ascorbic acid in avian systems has been for the most part neglected.
It is thought that ascorbic acid is metabolized in the same manner
as it is in mammalian species. The big difference in ascorbic
acid metabolism among mammalian species is seen in the excretion
products. Rats and guinea pigs oxidize the ascorbic acid molecule
to respiratory CO_2, while man excretes urinary oxalates.

It is well-known that stress, as manifested by unfavorable
environmental temperature, disease and crowding, causes a decline
in egg quality as measured by shell thickness, egg weight and
interior protein (Warren and Schnepel, 1940; Wilhelm, 1940).

Dietary supplementation of ascorbic acid to laying hens has
produced variable and inconsistent results on hen performance.

Thornton and Moreng (1958, 1959) were the first to show a possible beneficial effect of exogenous ascorbic acid on egg quality. Interior egg quality and shell thickness appeared to be improved. They noticed that the response to ascorbic acid was greater during the summer months. Perek and Kendler (1962, 1963) found ascorbic acid supplementation beneficial on egg production and egg weight, but non-significant in increasing shell thickness. Heywang and Kemmerer (1955), Hunt and Aitken (1962) and Arscott et al. (1962) did not find a beneficial effect in shell thickness or in general egg shell quality with ascorbic acid supplementation.

The effects of ascorbic acid on egg quality may be influenced by the types of diets employed. Thornton (1960a, b) reported an interaction between ascorbic acid and the calcium level on shell thickness, with responses appearing at the 2.5 and 3.0 percent levels in contrast to no response at the 2 percent calcium level. In addition, more consistent improvements have been obtained with rations containing 13 as compared with 17 percent protein.

Damage to the structure of the egg shell caused by climatic stress has received little attention. Mather et al. (1962) concluded from their work on the influence of environmental temperature on the microscopic structure of the egg that the quantity of matrix in the spongiosa was not influenced to the same degree as shell thickness. El-Boushy et al. (1968) studied the effects of heat stress and ascorbic acid supplementation on thickness, quality, structure and ultra-structure of the egg shell. Climatic stress significantly decreased the shell thickness and all its layers except the membranes. The favorable effect of ascorbic acid addition proved to be highly significant only for the thickness of the shell as a whole.

The use of controlled environmental temperature on laying hen performance has been studied. Ahmad et al. (1967) found that ascorbic acid supplementation was helpful in maintaining interior egg quality in White Leghorns as environmental temperatures increased from 21 ^{O}C to 29.4 ^{O}C and 35 ^{O}C. Egg shell thickness was also maintained during the increasing temperature. Lyle and Moreng (1968) also found that egg shell thickness was maintained with ascorbic acid supplementation when temperature was increased to 29 ^{O}C.

Nockels et al. (1968) presented data that withdrawal of ascorbic acid after prolonged supplementation depressed interior egg quality in eggs from hens which had been maintained at either optimum or 30.2 ^{O}C. Herrick and Nockels (1969) observed a significant increase in interior egg quality due to ascorbic acid supplementation. Eggs stored for two weeks maintained the higher egg quality when compared with the controls.

The influence of rapid changing environmental temperatures on the body temperature control of hens was studied by Thornton (1962). The effect of dietary ascorbic acid on body temperatures was recorded. Body temperature of both control and supplemented hens acclimated at 80 ^{O}F was increased when the ambient temperature was increased; but the increase was significantly greater in control individuals. A decrease in ambient temperature resulted in a greater fall in body temperature of the controls. These data indicated that ascorbic acid was of aid for maintaining body temperature. Ahmad et al. (1967), Lyle and Moreng (1968), Subaschandran and Balloun (1968) also presented evidence that ascorbic acid supplementation helps maintain body temperature during times of heat stress.

Squibb et al. (1955) studied the effects of the three diseases, coryza, cholera and Newcastle disease, on the level of serum ascorbic acid. Serum ascorbic acid decreased in birds infected with coryza, while it increased in the birds infected with cholera and Newcastle disease. This work presents evidence that ascorbic acid metabolism in chickens is disturbed by stress caused by disease.

Blood ascorbic acid levels in the chicken can be influenced by thyroid regulators (Thornton and Deeb, 1961). Their results showed that dietary iodinated casein caused an increase in blood ascorbic acid while thiouracil resulted in a decreased ascorbic acid level in the blood. It follows that the rate of ascorbic acid synthesis can be influenced by changes in the metabolic rate of the chicken.

Rumsey (1969) imposed a low level stress in hens to evaluate the "anti-stress" properties of ascorbic acid. Laying hens were subjected to simulated stress conditions by injection of ACTH or a synthetic corticosteroid, Dexamethasone. Both exogenous ACTH and Dexamethasone had a depressing effect on kidney ascorbic acid biosynthesis as well as blood ascorbic acid levels. Exogenous ascorbic acid also depressed kidney biosynthesis. Egg weight, egg shell thickness, and egg shell protein were not affected by the stress conditions imposed or by ascorbic acid supplementation.

In summary, it is clear that many factors may influence the ability to regulate ascorbic acid metabolism in avian species. At the present time, supplementation of ascorbic acid is not practiced in poultry feeding, but the evidence indicates that much may be learned by studying the physiological responses of chickens under various conditions when their diets are supplemented with ascorbic acid.

THE RECOMMENDED DIETARY ALLOWANCES FOR VITAMIN C

   Recommended Dietary Allowances are used as the basis for de-
cisions about the adequacy of food supplies, so it is important
that the basis for establishing them and their limitations be
clearly understood.

   Recommended Dietary Allowances are defined as "the levels of
intake of essential nutrients considered, in the judgment of the
Food and Nutrition Board on the basis of available scientific
knowledge, to be adequate to meet the known nutritional needs of
practically all healthy persons" (Food and Nutrition Board, 1974).
That is, they are recommendations for the quantities of a group of
specific chemical compounds that should be consumed by each member
of a population in order to provide reasonable assurance that the
physiologic needs of all will be met. This is a public health
concept. The RDA are nutritional standards. They are recommenda-
tions that can be used as the basis for practical decisions. They
are standards designed to be used in planning the food supply of
a population in order to ensure that the food procured will be
nutritionally adequate to maintain the health of that population.
They are standards also against which the nutritional adequacy of
the available food supply can be assessed in order to identify
possible nutrient shortages that could create public health prob-
lems.

   How are these standards established? The starting point is
the quantitative information about human requirement. Two types
of information are used for the most part: (1) information from
studies on human subjects in which the amount of nutrient required
to maintain satisfactory growth and body weight, to prevent de-
pletion of the nutrient from the body, to maintain some specific
body function or to prevent the development of unique signs or
symptoms has been established; (2) information from dietary surveys
indicating how much of the nutrient is consumed by populations
that are generally healthy and show none of the signs that are
associated with an inadequate intake of the nutrient. From the
first of these a figure for the average requirement of the popula-
tion studied can be obtained; from the second, an estimate of the
upper limit of human needs is obtained, but this may not be very
precise.

   But RDA are not average individual requirements, they must
obviously exceed average requirements if they are to cover the
needs of those with high requirements. Since individual require-
ments cannot be predicted, a statistical approach must be used
in estimating the amount by which the average requirement must be
increased to accomplish this. When information is insufficient
to permit a statistical prediction, selection of an appropriate
estimate involves judgment.

From information obtained in studies of human subjects, an
estimate of variability among the individuals studied can be cal-
culated. Assuming that requirements follow a normal distribution
pattern, 97.5% of the population should have requirements below
the mean plus twice the coefficient of variation. This amount,
the average plus twice the coefficient of variation, provides an
estimate for a standard that should cover the needs of most of
the population, provided the nutrient is utilized with high effi-
ciency. However, if efficiency of utilization of the ingested
nutrient is known to be low, owing for example, to incomplete
absorption, the recommended level of intake must be increased fur-
ther to allow for this. The value obtained after allowing for
incomplete absorption should meet or exceed the needs of the vast
majority of individuals.

It is important to emphasize that RDA do not take into con-
sideration the losses of nutrients during the processing and prep-
aration of foods, nor are the RDA designed to cover increased needs
resulting from severe stress, disease, or trauma above the usual
minor infections and stresses of everyday life. The processing
losses must be allowed for separately in planning a food supply,
and the increased needs represent clinical problems that must be
given individual consideration.

Nutritionists agree among themselves about the amounts of
each essential nutrient that should be consumed daily. This gen-
eralization, however, does not apply to vitamin C or ascorbic acid
which continues to be controversial. This controversy centers
around two major topics. The first is how much ascorbic acid
should be recommended "to meet the known nutritional needs of prac-
tically all healthy persons" and the second is what are the effects
of giving very large amounts of ascorbic acid?

As a result of this controversy, most persons (and a great
many physicians) are confused about the daily allowance of vitamin
C needed to support optimal health. In attempting to clarify this,
two basic concepts should be acknowledged. First, on the basis
of available scientific evidence, the Committee on Dietary Allow-
ances of the National Academy of Sciences National Research Council
establishes Recommended Dietary Allowances which in the judgment
of its members provide adequate intakes for practically all
healthy persons. Secondly, there is a vast difference between
"physiologic" amounts of any nutrient and "pharmacologic" doses
which may be given for various medically indicated reasons. The
lowest range, representing the amounts needed or recommended to
prevent signs or symptoms of deficiency in practically all healthy
persons can be termed the "physiologic dose" (the RDA level, 45 mg
of vitamin C/day). Somewhat larger amounts (100-2000 mg/day),
generally in the range of ten times the physiologic dose, may be
used to treat an illness or condition quite unrelated to the

generally recognized manifestations of deficiency of that nutrient.
This level of intake can be termed "pharmacologic dose". Very
large intake of an essential nutrient, for example 100 times (or
more) the physiologic dose, may induce undesirable or toxic signs
and symptoms, and can be called "toxic dose" (2000-4000 mg/day).
It is not uncommon for pharmacologic doses to cause deleterious
side reactions (Barnes, 1975).

With the understanding of the above concepts, it is safe to
recommend the following levels for vitamin C intake. As little as
10 mg ascorbic acid will prevent scurvy. This level may be re-
garded as a minimum requirement but it does not ensure fully sat-
isfactory tissue levels. Each day the adult male removes about 30
mg ascorbic acid from body stores. The recommended allowance has
been set at 45 mg for males and females over 11 years, 35 mg for
infants, 40 mg for children, and 60 mg for pregnancy.

During infections such as tuberculosis, rheumatic fever, and
pneumonia and severe stress such as burn injuries the ascorbic
acid recommended allowance should be increased.

Fruits and vegetables are excellent sources of vitamin C.
Table 13 illustrates the vitamin C content in some selected foods.
Like other water soluble vitamins, ascorbate is labile to heat and
oxygen. Thus, fresh fruits and vegetables, including juices, are
excellent dietary sources in meeting RDA.

Since vegetable cells contains an enzyme called ascorbic oxi-
dase, cutting vegetables finely causes a greater quantity of re-
lease of this enzyme which causes more breakdown of vitamin C.
The rate of ascorbic oxidase activity increases as the temperature
is raised; thus, gradual heating of vegetables destroys ascorbate.
Boiling causes a breakdown of the enzyme. The loss of vitamin C
due to ascorbic oxidase can be minimized by immersing the vegetables
directly in boiling water which causes immediate destruction of the
enzyme. Vitamin C is more readily destroyed than other vitamins.
During cooking, ascorbate loss occurs as a result of (1) cutting
the vegetables finely; (2) using an excess of water which leaches
the vitamin C out of the cells; (3) gradually heating the vegetables
rather than putting them in boiling water; (4) overcooking; (5)
adding sodium bicarbonate in order to preserve the color of the
vegetables as alkalinity destroys ascorbate.

TOXIC EFFECTS OF LARGE VITAMIN C INTAKE

Gastrointestinal Disturbances

Gastrointestinal disturbances are perhaps the most consistent
abnormalities noted following the ingestion of large quantities of
ascorbic acid. Nausea, abdominal cramps, and diarrhea are

Table 13. Vitamin C Content in Some Selected Foods

Item	Vitamin C (mg/100 g edible portion)
Acerola (Barbados Cherry)	1,300
Apples (raw)	4
Apricots (raw)	10
Avocados (raw)	14
Bananas (raw)	10
Beans, Lima (cooked)	17
Beans, snap (cooked)	12
Beef	0
Broccoli (cooked)	90
Cabbage (cooked)	24
Carrots (raw)	8
Collards (cooked)	46
Corn, sweet (cooked)	9
Currants, Black European (raw)	200
Eggs	0
Eggplant (cooked)	3
Fish	0
Grapefruit, pulp	38
Grapefruit, juice unsweetened	34
Lemon, Juice	45
Lettuce (raw)	15
Milk, cow	1
Muskmelons (raw)	33
Oranges (raw)	55
Oranges, Juice (canned)	40
Peaches (raw)	7
Pears (raw)	4
Peas, green (cooked)	20
Pineapple (raw)	17
Pineapple (juice)	9
Pork	0
Poultry	0
Potato (cooked)	20
Spinach (raw)	51
Spinach (cooked)	28
Summer squash (cooked)	10
Strawberries (raw)	59
Tomato, ripe (raw)	23
Tomato juice	16
Wheat flours	0

[a]from Watt and Merrill (1963)

frequently mentioned. These may be due to the ascorbic acid itself
or to sensitization reactions such as hives, angioneurotic edema,
and skin rashes, which have also been reported in some of these
patients. These effects may be ameliorated or eliminated by taking
the ascorbic acid as a buffered salt or after meals.

Renal Stone Formation

One of the feared consequences of excessive ascorbate intake
is the possible development of renal stones or nephrocalcinosis.
Stone formation may be accelerated with the administration of large
doses of ascorbate. In those who are prone to renal stone forma-
tion, stones may form more easily. This includes persons with
oxalosis, hyperuricemia, and cystinuria.

Oxaluria-Oxalosis

Studies on the sources of oxalic acid indicate that in the
normal subject approximately 50% of the daily excretion of oxalic
acid, about 50 mg, is derived from dietary ascorbate. The source
of the other 50 mg varies. According to Lewin (1973), the total
intake of vitamin C should be kept below 4 grams daily because of
the increasingly unacceptable levels of oxalate ions that are likely
to accompany the high ascorbate intake.

VITAMIN C DEFICIENCY

Scurvy

Scurvy is produced by prolonged deficiency of vitamin C.
Human volunteers deprived of vitamin C showed no clinical manifes-
tations for 17 weeks. The first signs noted are hyperkeratotic
hair follicles from 17 to 21 weeks, followed by perifollicular
hemorrhages at 26-34 weeks (Medical Research Council, 1948). Since,
in ordinary circumstances, an infant or adult is not completely
deprived of vitamin C, it can be assumed that at least 6-9 months
would elapse before clinical scurvy appears even with gross de-
ficiency. The basic pathological change in scurvy is defective
formation of collagen. The lining of blood vessels is defective
because the cells are not cemented together. The bony matrix or
framework is poorly formed for the same reasons.

Subclinical Scurvy

Before the development of clinical signs of scurvy, vague
general symptoms such as lassitude, fatigue, weakness, irritability,
a tendency to recurrent infections and aching of bones may be noted
(Hodges, 1969).

Clinical Scurvy

Clinical symptoms appear when the body pool of vitamin C de-
creases to about 300 mg (Hodges, 1969). Clinical scurvy is mani-
fest by changes in: (1) blood vessels; (2) skin; (3) bones;
(4) teeth and gums; (5) anemia; and (6) general symptoms.

Blood vessels. The blood vessels become fragile and porous
due to defective formation of collagen in the lining of the wall.
This leads to spongy gums, hemorrhages into the skin, seen as
petechiae and ecchymoses, and hemorrhages into the alimentary and
urinary tract. Conjunctival hemorrhages occur by 74-95 days in
experimental human scurvy. Muscular and subperiosteal hemorrhages
may also occur.

Skin. The skin becomes rough and dry. Early hyperkeratotic
changes are seen in the hair follicles, and are most marked on
legs and buttocks. The occurrence of petechial hemorrhages around
hair follicles and larger hemorrhages, ecchymoses, have already
been referred to.

Bones. During normal growth, cartilage cells at the growing
end of bones proliferate. These are invaded by osteoblasts to
form the bony matrix, also known as osteoid tissue. Ossification
occurs when calcium salts are deposited in this matrix, forming
bone.

In the absence of vitamin C the formation of bony matrix (os-
teoid tissue) is defective. Instead of osteoblasts, fibroblasts
develop and bone formation is retarded. This gives rise to osteo-
porosis and spontaneous fractures. The periosteum is not attached
firmly to the bone and subperiosteal hemorrhages occur, usually
at the lower end of the femur and upper end of the humerus. These
bony changes are found in infants and children with scurvy.

Teeth and Gums. Deficiency of vitamin C in children leads
to defective formation of the teeth. The dentine becomes porous,
the alveolar bone is absorbed and the teeth may fall out. The
gums become spongy and bleed easily if slight pressure is applied.
Hypertrophy of the gums tends to bury the teeth and infection and
ulceration of the unhealthy gums is common. These changes in the
gums may not be seen in an edentulous subject.

Anemia. Microcytic hypochromic anemia is a common feature
in scurvy. The anemia is partly due to deficient absorption of
iron, with a low intake of vitamin C and partly due to blood loss
from hemorrhages. Deficiency of vitamin C also disturbs folic
acid metabolism and a megaloblastic anemia may develop.

General Manifestations. Scurvy is manifested by pyrexia, rapid pulse and susceptibility to infection. Wound healing is delayed.

Aspirin is more likely to produce gastric mucosal bleeding in a vitamin C-deficient subject.

METHODS OF ASSESSING VITAMIN C ADEQUACY

A person's nutritional status, including adequacy of vitamin C, may be determined on the basis of clinical signs of a dietary deprivation, biochemical measurements, or dietary intake findings. Information on the dietary intakes of ascorbic acid can be useful in the general assessment and prediction of the nutritional status of a population, but usually such data cannot provide the desired direct knowledge as to the vitamin C nutritional status of the individuals. Even the presence of clinical signs associated with a vitamin C deficiency may not always be a reliable indicator of a deficiency of the nutrient, since such signs may be the result of local infections or improper oral hygiene. Therefore, for the most part, biochemical measurements represent the most objective assessment of the vitamin C nutritional status of an individual.

Healthy adult men have an average body pool of 1.5 grams of ascorbic acid. In experimental vitamin C deficiency, scurvy developed when the body pool of ascorbic acid had been depleted to a level of 300 mg or less (Baker et al., 1971). Occurred in the plasma and whole blood ascorbate levels. The fall in plasma ascorbic acid appears to be more pronounced than the reduction in the ascorbate body pool. Consequently, a low plasma ascorbate level may not necessarily indicate scurvy, but scurvy will invariably ensue if the absence of ascorbic acid in the serum persists. Moreover, continued serum ascorbic acid levels of less than 0.10 mg/dl would probably eventually lead to signs of scurvy. Such was observed in the study of Hodges et al. (1971) where the first signs of scurvy appeared when the serum ascorbic acid levels ranged from 0.13 to 0.24 mg/dl. There was also a definite relationship between the body reserves of ascorbic acid and whole-blood ascorbate levels. Signs of scurvy were observed when the whole-blood ascorbate level fell below 0.3 mg/dl. At this point, the body pool of ascorbic acid had fallen from an initial normal level of approximately 1500 mg to a pool size ranging from 96 to 490 mg.

As noted previously, serum (or plasma) ascorbate levels may not always fully reflect vitamin C intake or the state of the body ascorbate reserves. Nevertheless, within a relatively limited range, serum levels of ascorbic acid show a linear relationship with the intake of vitamin C. With a given intake of the vitamin, the serum ascorbic acid concentration will plateau at a given level, around 1.4 mg/dl. We have observed this when subjects were given

two doses of 500 mg of vitamin C, one in the morning and one in
the evening. When serum vitamin C levels fall below 0.2 mg/dl,
signs of scurvy are commonly observed. With an intake of 45 g
of vitamin C per day, representing the 1974 Recommended Dietary
Allowance, a serum ascorbate level of approximately 0.6-0.8 mg/dl
could be expected. Higher serum ascorbate levels may be obtained
with greater intakes of vitamin C. However, the maximal serum
ascorbic acid level appears to be at about 1.4 mg/dl, at which
point renal clearance of the vitamin rises sharply. The current
practice of some people to ingest high doses of vitamin C can
result in the temporary attainment of higher serum ascorbic acid
concentrations.

The Interdepartmental Committee on Nutrition for National
Defense (ICNND) has considered serum ascorbic acid levels of less
than 0.10 mg/dl "deficient" and levels from 0.10 to 0.19 mg/dl as
"low", while levels of 0.20 mg/dl and above are termed "acceptable"
(Manual for Nutrition Surveys, 1963). In the nutrition survey of
Canada (Nutrition Canada National Survey, 1973) subjects with serum
vitamin C levels of less than 0.2 mg/dl were considered at "high
risk". Levels of 0.6 mg/dl to 0.4 mg/dl were considered necessary
for subjects 19 years of age or younger and for subjects 20 years
and above, respectively, to be considered at "low risk" with re-
spect to vitamin C nutriture. No differences were considered with
respect to the sex of the subjects.

The results of the studies of Hodges et al. (1971) indicated
that signs of scurvy may be encountered in subjects with serum
ascorbate levels below 0.20 mg/dl and would support the guidelines
employed in the Canadian nutrition survey. Based on the above
data, a revision of the guidelines interpreting serum vitamin C
data has been suggested by Sauberlich (1975) as seen in Table 14.

Leukocyte ascorbic acid concentrations have been considered
to be more closely related to tissue stores of the vitamin than

Table 14. Guidelines for the Interpretation of Vitamin C
           Levels (mg/dl)[a]

Measurement	Acceptable	Low	Deficient
Whole Blood Vitamin C	>0.50	0.30-0.49	<0.30
Serum Vitamin C	>20	0.10-0.19	<0.10
Leukocyte Vitamin C	>15	8-15	0-7

[a]Adapted from Sauberlich, 1975.

serum concentration (Sauberlich, 1975, Sauberlich et al., 1973,
1974). Others have suggested that leukocyte ascorbic acid concen-
trations do not reliably reflect tissue status and that both serum
and leukocyte ascorbate concentrations should be used to do this
(Loh, 1972, Keith and Pelletier, 1974). In general, serum vitamin
C levels tend to respond more readily than leukocytes to recent
dietary intake of ascorbic acid. However, low serum ascorbate
concentrations do indicate low or inadequate intakes of vitamin C
with probably only partial reserves present. Nevertheless, under
controlled intakes of ascorbic acid, there is a relationship be-
tween ascorbate intake and ascorbate levels in both the leukocytes
and serum. With an intake of 45 mg of vitamin C per day (RDA),
leukocytes will contain approximately 20 mg/dl. Subjects with
leukocyte ascorbate levels of less than 8 mg/dl are considered to
be at "high risk". Concentrations may fall to zero in clinical
cases of scurvy (Sauberlich, 1975).

Although numerous procedures and modifications have been re-
ported for the determination of vitamin C in white blood cells,
the methods must still be considered technically difficult and
require relatively large blood samples. Consequently, present
procedures are not practical for routine use in nutrition surveys.
With adequate laboratory facilities, measurement of leukocyte
ascorbate levels can be a useful diagnostic technique for clinical
cases of vitamin C deficiency. To some extent measurement of serum
ascorbate levels have been favored over measurement in white blood
cells because of the greater percentage increase in serum ascorbate
concentrations in response to graded intakes of vitamin C. Such
factors, along with the relative ease of measurement, have led to
the common use of serum ascorbate determinations for assessing
vitamin C nutritional status. This approach was used in national
nutrition surveys of Canada and the United States.

Urinary vitamin C excretion data have received only limited
use in determining the nutritional status of this vitamin. As
a result, guides for the interpretation of urinary vitamin C levels
in terms of nutritional status have not been established. Analy-
tical procedures for measuring ascorbic acid in urine are more
difficult and less reliable than those used for measuring the vi-
tamin in serum. Nevertheless, in the scorbutic patient, the
urinary excretion of vitamin C would be expected to be essentially
zero and hence, could provide supportive diagnostic information.

On occasion, ascorbic acid saturation tests are used to obtain
information as to tissue ascorbate deficit in individual patients
(Sauberlich et al., 1973, 1974; Dutra DeOliveira et al., 1959;
Lowry, 1952). For nutrition surveys such tests are not practical
and are probably of little use. At best, the saturation or load-
ing test must be conducted with considerable care and the results
interpreted with caution. However, for clinical cases the test

may be of conclusive value in excluding scurvy as a diagnosis and of considerable usefulness in obtaining the diagnosis of scurvy.

The various analytical procedures used to measure ascorbic acid are summarized by Sauberlich (1975). The method of Roe and Kuether (1943) has generally been considered the "classical technique" for vitamin C quantitation.

SUMMARY

In summary, it is obvious that considerable interest and research has developed in the area of ascorbic acid metabolism. The biochemist has contributed much information concerning the identification and detailed study of enzyme systems and related nutrients on metabolites that control the synthesis, functions and end products of vitamin C in living organisms. Further studies are needed on the role of antihistamine effect of ascorbic acid, vitamin C and bile acid metabolism, vitamin C and the common cold, ascorbic acid and bioenergetics, vitamin C and prevention of carcinoma, ascorbate and ascorbate-SO_4 metabolism.

Furthermore, reports on the effects of massive intakes of vitamin C have raised many questions and violently different opinions. Most of the claims for dramatic benefit from very high intakes have been based on resistance to or therapy toward the common cold and on quantities very far above the amount apparently needed for normal reproduction, lactation, early growth, and longevity. Very few physicians with extensive experience in this type of research recommend the practice of using large dosages, such as 1 to 20 g per day.

Hence, we have a situation where many experienced nutrition scientists trained as biochemists or physicians and in other specialized branches of science are not in agreement on either of two major issues:

   a. Should the quantities recommended for daily consumption
       of vitamin C be those adopted in the table of Recommended
       Dietary Allowances by the National Academy of Sciences?
       Differences in opinion on this quantity are not very
       great -- seldom more than a factor of twice the values now
       used.
   b. What should be the recommended intake of vitamin C
       with special reference to prevention or therapy of the
       common cold or other disease risks to which the public
       is exposed?

The Food and Nutrition Board has adopted a consensus of views among well-trained clinical biochemists and physicians in arriving at the recommended daily intake, which ranges from 35 to 80 mg per

day, depending on age and including an extra allowance for lacta-
tion. There is an increasing concern that the recommendation for
growing children and adults should be higher than the present stated
values of 40 to 45 mg per day, perhaps in the range of 70 to 75 mg,
and 100 mg during lactation.

The area of most intense controversy currently is in regard
to the quantity that should be recommended for consumption by the
public without further consideration when there is an indication of
an oncoming cold. Quantities in the range of 5 to 10 g or more
per day are sometimes recommended by laymen and some scientists.
A few enthusiastic laymen and some physicians suggest practices
such as a regular intake in the range of 1 g or more each day.
Claims are made that these very large intakes are harmless. Others
with excellent research training and much experience in medicine,
public health, and biochemistry believe that such large intakes
have little if any significant value and entail significant hazards
to health, such as development of kidney and bladder stones, and
alterations in carbohydrate metabolism. Thus far there has been
little evidence to indicate a mechanism to account for the claims
made in supporting the high dosages, and very little research has
been reported to compare the value of vitamin C with other potential
drugs such as antihistamines. Thus, until these questions are
clarified by further studies, a conservative approach should be
taken in terms of recommending large amounts of vitamin C.

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ACKNOWLEDGEMENTS

VITAMIN A AND RETINOL BINDING PROTEIN ALTERATIONS IN DISEASE

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VITAMIN A

Historical Developments

A lipid-soluble compound which is essential for life and present in egg yolk, was first described by Stepp (1909). A similar compound was later found in butterfat, egg yolk, and cod liver oil and was named "fat soluble A" by McCollum and Davies (1913, 1915) to distinguish it from essential water-soluble nutrients, which they called "water soluble B". The "fat-soluble A" factor was capable of preventing xerophthalmia (McCollum and Simmonds, 1917) and night blindness (Fridericia and Holm, 1925). Drummond (1920) named the active lipid "vitamin" A.

A growth-promoting substance in plant extracts was found by Steenbock et al. (1921). After Karrer et al., (1930) elucidated the structure of β-carotene and that of retinol (Karrer et al., 1931, 1933), the provitamin role of β-carotene became obvious.

Holmes and Corbett (1937) succeeded in crystallizing vitamin A from fish liver. Wald (1935 a, b, c, 1936 a, b) isolated the chromophore from bleached retinas. Morton (1944) and Morton and Goodwin (1944) demonstrated that the chromophore was retinal. Arens and Van Drop (1946) and Isler et al., (1947) succeeded in achieving the chemical synthesis of pure vitamin A. Shortly thereafter, the total synthesis of β-carotene was also reported (Karrer and Eugster, 1950).

Nomenclature and Chemistry

    The nomenclature follows the rules on Biochemical Nomen-
clature (IUPAC, 1960;   IUPAC-IUB, 1966). The parent compounds
retinol, retinal and retinoic acid (Figure 1) are the most
frequently involved in vitamin A metabolism. The synthetic
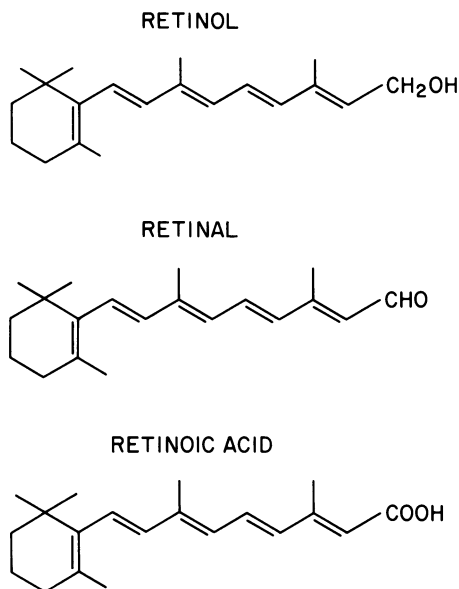
RETINOL

RETINAL

RETINOIC ACID

Fig. 1. The structures of retinol, retinal and retinoic acid.

chemistry of vitamin A and derivatives have been extensively
reviewed (Isler et al., 1967, 1970; Schwieter and Isler, 1967).
A review of synthetic processes for obtaining β-carotene and
carotenoids has appeared (Mayer and Isler, 1971). Vitamin A is
a generic term used for all compounds, other than carotenoids,
that exhibit qualitatively the biological activity of retinol.
The term "retinoids" in recent years has been accepted as a
general term that includes both the natural forms of vitamin A
and synthetic analogs. Vitamin A is necessary for the growth,
health and life of higher animals. Animals without vitamin A
will cease to grow, and in time die. Vitamin A is necessary for
vision, reproduction, maintenance of differentiated epithelia and
for mucus secretion. One International Unit of vitamin A is
defined as 0.3 µg of all-trans retinol. Generally, 1 µg of
retinol is assumed to be biologically equivalent to about 6

μg of β-carotene, or about 12 μg of mixed dietary carotenoids.

Absorption

Vitamin A is obtained in the diet primarily as long-chain
fatty acid esters of retinol. The intestine hydrolyzes dietary
retinyl esters, and the resulting retinol is then absorbed into
the mucosal cell. The newly absorbed retinol in the mucosol cell
or retinol newly synthesized from carotene is re-esterified with
long-chain, mainly saturated, fatty acids and incorporated into
lymph chylomicrons (Huang and Goodman, 1965). The chylomicrons
are absorbed in the lymph and enter the circulation when the
lymphatic vessels join the blood stream. Most of the chylomicron
triglyceride is removed from the blood stream by extrahepatic
tissues. The remaining chylomicrons are smaller (Redgrave, 1970)
and contain virtually all of the chylomicron retinyl esters which
are almost entirely removed from the circulation by the liver
(Goodman et al., 1965; Redgrave, 1970). In the liver, hydrolysis
and re-esterification occur, and the resulting retinyl esters
(mainly retinyl palmitate) are stored within hepatocytes asso-
ciated with lipid droplets.

Biosynthesis

β-carotene is converted to vitamin A primarily in the
intestinal mucosa. Two enzymes are involved in the biosynthetic
processes: first, β-carotene-15, 15'-dioxygenase, and second,
retinaldehyde reductase. β-carotene-15, 15'-dioxygenase cata-
lyzes the cleavage of β-carotene at the central double bond to
yield two molecules of retinaldehyde (Goodman and Olson, 1969).
The cleavage reaction appears to occur by a dioxygenase mechanism
(Goodman et al., 1966; Goodman and Olson, 1969).

The reduction of the newly formed retinaldehyde to retinol
is catalyzed by another soluble mucosal enzyme, retinaldehyde
reductase (Fidge and Goodman, 1968). This enzyme has a molecular
weight in the range of 60,000-80,000, and has a requirement of
NADH or NADPH. Retinaldehyde reductase appears to be a relatively
nonspecific aldehyde reductase for several short- or medium-chain
aliphatic aldehydes.

Functions

Night blindness results from vitamin A deficiency, because
the vitamin is required to regenerate the chemical which allows
us to see in dim light. Rhodopsin is the complex of opsin (a
protein present in the rods of the eye) and the retinal form of
vitamin A. The simplified process is shown in Figure 2. A
supply of vitamin A is needed to regenerate the complex, because
some retinal is degraded in the process of rhodopsin degradation

caused by bright light. Normal vision cannot be quickly obtained
after rhodopsin bleaching by bright light, such as that from an
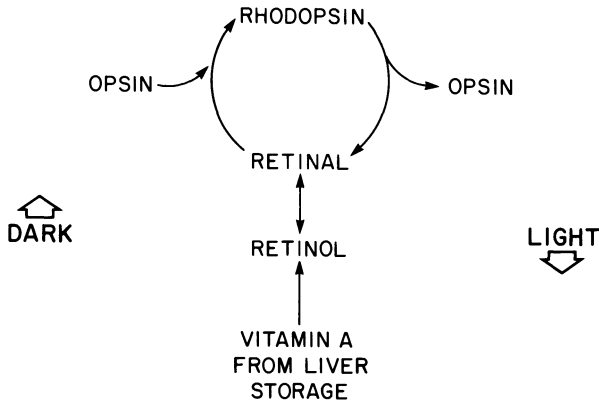oncoming car, unless there is an ample supply of vitamin A.

Figure 2. Vitamin A function in the visual cycle.

Only retinol and retinal can be used for maintenance of the
visual cycle, whereas retinol, retinal and retinoic acid function
in maintaining growth. Rats fed retinoic acid maintained their
growth rate, but soon developed eye lesions.

Vitamin A is also required for the maintenance of epithelial
tissue; that is, cells found in the outer layers of the skin as
well as lining the gastrointestinal, respiratory and urogenital
tracts. Vitamin A deficiency results in a transformation of
epithelial cells from soft, moist tissue to cells which are hard
and dry, or keratinized. This effect has been related to the
protective effect of vitamin A in maintaining the integrity of
epithelial tissue against infective organisms, particularly of
the respiratory tract. The biochemical effect of vitamin A in
this function may directly effect the synthesis of mucopoly-
saccharides and protein. DeLuca et al., (1975) found that
labeled mannose incorporation into liver glycoproteins in both
mildly and severely vitamin A deficient hamsters decreased by
about 70% compared to pair-fed control animals.

Some research suggests that vitamin A allows the maturation
of osteoblasts to osteoclasts, a conversion necessary for bone
remodeling and therefore for continued bone growth. Mellanby
(19744) likened vitamin A to "the director of building operations
in the bone" and concluded that it might be expected that in
vitamin A deficiency that the osteoblasts and osteoclasts would

either cease to work or work in a completely disorderly way.
This result was indeed obtained in fowl (Howell and Thompson,
1967 a,b). Avitaminosis A affected the nervous system indirectly
by causing an overgrowth of the periosteum. Similar findings
were obtained in fowl in which deficiency of vitamin A was
precipitated suddenly by removal of retinoic acid from the diet.

Likewise, in tooth development vitamin A is necessary for
the normal differentiation and function of the ameloblasts, cells
of ectodermal origin which are responsible for the formation of
dental enamel.

Evans and Bishop (1922) found that female rats fed diets low
in vitamin A suffered from prolongation of estrus and failed to
enter diestrus; a vaginal smear showed the presence of cornified
cells. The rats also failed to ovulate. Administration of
butterfat or cod liver oil readily reversed these changes within
1-3 days. One hundred days after placement on a low vitamin A
diet, only 22% of rat matings resulted in the birth of living
young. Implantation of the sperm into the egg generally did not
occur, indicating that the sperm did not reach the egg because of
the keratinized surface of the vaginal epithelium (Evans, 1928).

Evans (1932) and Mason (1932, 1933) showed that rats fed
diets containing adequate amounts of vitamin E but deficient in
vitamin A cause degeneration of the germinal epithelium in the
testes and absence of spermatozoa in the epididymal fluid.
Vitamin A can reverse the changes resulting from its deficiency.
Howell et al., (1963) found that depletion of retinol in rats
caused a basic lesion at a state in spermatocytogenesis before
the meiotic division of the spermatocytes to spermatids. Similar
results were obtained in the guinea pig (Howell et al., 1967).

Vitamin A also has a known effect on lysosomes and their
enzymatic contents. Hypervitaminosis A reduces the stability of
rat liver lysosomes and increases the release of acid hydrolases
from these subcellular particles (Anonymous, 1966).

RETINOL BINDING PROTEINS

Role in Transport of Vitamin A

In the 1960's vitamin A was shown to be transported in
plasma as the alcohol, retinol, bound to a specific transport
protein, retinol-binding protein (RBP) (Kanai et al., 1968).
Human RBP is a single polypeptide chain with a molecular weight
of about 21,000 with a single binding site for one molecule of
retinol. In plasma, most of RBP normally circulates as the
retinol-RBP complex. The usual concentration of RBP in plasma is
about 40-50 µg/ml.

RBP interacts strongly with prealbumin, and normally circulates as a 1:1 molar RBP-prealbumin complex. Evidence suggests that specific receptors for serum RBP are present on the surface of certain vitamin A-requiring target cells (Heller, 1975; Rask and Peterson, 1976). RBP may deliver retinol to target tissues specifically at these locations. The retinol then presumably enters the target cell, where it may become associated with the intracellular cytosol binding protein for retinol (CRBP). The complex of retinol with CRBP may be the form in which retinol is transmitted to specific sites within the cell, and may be involved directly in the subsequent metabolism and/or biochemical functions or retinol within the cell. The intracellular binding proteins differ from plasma RBP with regard to molecular weight, immunoreactivity, binding affinity for serum prealbumin, and ultraviolet and fluorescence spectral characteristics.

Clinical Studies of RBP

Even though RBP has been studied in many diseases to date, no disease has been found where RBP was totally absent or has had abnormal immunological properties. In patients with liver disease, the plasma concentration of vitamin A, RBP and prealbumin were all found to be markedly decreased (Smith and Goodman, 1971). These low plasma concentrations of RBP and prealbumin may reflect a reduced rate of synthesis of the proteins by the diseased liver. Patients with liver disease and low plasma RBP concentrations (below 20 µg/ml) were found to have impaired dark adaptation (Vahlquist et al., 1978). This study suggests that these patients had peripheral vitamin A deficiency symptoms secondary to their inability to mobilize vitamin A from the liver. In these patients, vitamin A therapy did not affect either the reduced dark adaptation ability or the low plasma RBP concentrations. In another study (Russell et al., 1978) vitamin A therapy of patients with alcohol-associated cirrhosis and marginal vitamin A status appeared to stimulate RBP release from the liver, and led to improvement in dark adaptation.

In patients with chronic renal disease, the plasma concentrations of RBP and of vitamin A were greatly elevated, while that of prealbumin remained normal (Smith and Goodman, 1971). The elevated concentrations of RBP reflect the fact that the kidney is normally the main catabolic site for RBP, so that RBP metabolism is impaired in chronic renal disease. RBP is small enough to be filtered by the renal glomeruli, whereas prealbumin and the RBP-prealbumin complex are not. Very little RBP is normally present in the free state. Its glomerular filtration and renal metabolism are sufficiently large to constitute RBP's major catabolic route. The important role of the RBP-prealbumin complex in sparing RBP from glomerular filtration was shown by turnover studies demonstrating a much higher fractional turnover

rate for free RBP than for RBP bound to prealbumin (Vahlquist et al., 1973). Very little RBP appears in the urine normally, as nearly all of the filtered RBP is resorbed and degraded by the renal tubules. In contrast, patients with tubular proteinuria clear RBP from plasma at a normal rate, but excrete large amounts of RBP in the urine.

VITAMIN A AND RETINOL BINDING PROTEINS AND CANCER

Tumor Cell RBP's

Some tumors contain both CRBP and CRABP (cytosol binding protein for retinoic acid), some only CRABP and in some none of these proteins have been detected (Ong and Chytil, 1976). A positive correlation between the responsiveness to vitamin A treatment and the presence of CRABP has been observed.

Specific antibodies against cellular retinol-binding protein, raised in rabbit, were detected by sucrose gradient centrifugation and gel filtration using tritium-labeled, CRBP prepared by reductive methylation. By means of a radioimmunoassay, pure CRBP from liver and testis as well as CRBP present in a crude extract of liver were compared. All preparations showed identical immunoreactivity, suggesting CRBP is not tissue-specific (Ong and Chytil, 1979).

Vitamin A and Cancer

Rowe and Garlin (1959) investigated the effect of vitamin A deficient diet on oral carcinogenesis induced by 7,12-dimethyl-1,2-benzantracene (DMBA) in golden hamsters. A 5% solution of carcinogen was applied twice weekly for 13 weeks. Neoplastic malignant lesions in hamsters given the vitamin A deficient diet were about 30% greater than the supplemented hamsters. Levij and Polliak (1968) found that topical vitamin A as retinyl palmitate applied to hamster and cheek pouches with DMBA for 12 weeks caused an increase in the size of squamous cell carcinomas. Chu and Malmgreen (1965) compared the incidence of neoplastic lesions of the alimentary tract of Syrian golden hamsters after oral feeding of DMBA alone or in combination with retinyl palmitate and benzopyrene alone or in combination with retinyl palmitate. Vitamin A had marked inhibitory effect on stomach carcinomas.

Saffiotti et al., (1967), Cone and Nettesheim (1973) and Smith et al., (1975 a,b) found that vitamin A prevented benzopyrene induced respiratory squamous cell carcinoma in animals.

Mouse skin papillomas can also be prevented by oral administration of 100 IU of vitamin A per gram of food (Davies, 1967). Shamberger (1971) has also reduced DMBA-croton oil induced mouse skin papillomas by applying vitamin A with the carcinogen to the skin. Bollag (1971) has induced mouse skin tumors with DMBA and croton oil. When papillomas reached at least 4 mm in diameter vitamin A (either as retinyl palmitate or as retinoic acid) was given orally or intraperitoneally. After 2 weeks of treatment with retinoic acid, 60-80% regression of the papilloma volume was estimated. Similar results were obtained with oral administration of retinoic acid or retinyl palmitate. Bollag (1972) has also given mice retinoic acid (200 mg/kg every 14 days) orally during the promotion phase of carcinogensis. The volume of papillomas was greatly reduced and the appearance of both papillomas and carcinomas as delayed. Bollag and Ott (1971) used retinoic acid as a chemotherapeutic agent to regress human skin basal cell carcinomas.. Verma et al., (1978) has observed that retinoids inhibit 12-0-tetradecanoylphorbol-13-acetate induced ornithine decarboxylase activity in mouse epidermis. There is some evidence that this phenotypic change is an essential component of the mechanism of skin tumor promotion.

Smith et al., (1972) have transplanted fetal lung tissues mixed with 20-methylcholanthrene (MCA) into the thigh muscle of Balb/c mice. The animals were placed on deficient, normal and excess retinyl palmitate diets. Carcinomas arose in the adequate or deficient groups but no carcinomas were observed in the group fed high vitamin A levels.

Prostate glands of mice were grown in tissue culture for 7-9 days. Methylcholanthrene caused hyperplasia of the alveolar epithelium, with subsequent squamous metaplasia or parakeratosis (Lasnitzki and Goodman, 1974). β-Retinol, β-retinoic acid, α-retinoic acid and cyclopentenyl analogue inhibited the effects of methylcholanthrene.

Vitamin A enhances the anti-tumor effect of cyclophosphamide in mammary gland adenocarcinoma in rats and mice (Anton and Brandes, 1968) and of 1,3-bis (2-chloro-ethyl-1-nitrosourea; (BCNU) (Cohen and Carbone, 1972). Synthetic retinoids can prevent DMBA-induced mammary cancer (Moon et al., 1976).

Hypervitaminosis A prevented the appearance of bladder squamous metaplasia and squamous cell neoplasia in rats fed (N-4-(5-Nitro-2 furyl)-2-thiazolyl formamide (Cohen et al., 1976). Feeding of 13-cis-retinoic acid after completion of carcinogen treatment diminished the number and severity of tumors induced in the bladders of male Fischer rats by 12 oral doses of N-butyl-N-(4-hydroxy-butyl) nitrosamine (Grubbs et al., 1979). Similar results have been observed in C57BL/6 mice (Becci et al., 1978).

The growth of Shope rabbit papilloma has been inhibited by hypervitaminosis A (McMichael, 1965). Vitamin A has also reduced the tumor size of mice innoculated with a murine sarcoma virus of the Maloney strain (Levine et al., 1975). Retinoic acid has shown an inhibitory effect on the growth of untransformed, transformed and tumor cells in vitro (Lotan and Nicolson, 1977). Some carcinogenesis test systems have not been effected by vitamin A.

Vitamin A Toxicity and Cancer Prevention

The toxic effects of vitamin A may limit its effective use in human cancer prevention. Sporn et al., (1976) have summarized the effectiveness of retinoids. These derivatives of vitamin A have the same anticarcinogenic effect as vitamin A but are not as toxic as vitamin A. For example, retinyl methyl ether is 20 to 100 times less toxic than retinol or retinoic acid in their comparative toxicity to tracheal cartilage in organ culture. Because of their lesser toxicity vitamin A anticancer experiments in animals mostly utilize retinoids in their experimental design.

Need for Further Research

A large clinical trial would be useful to test retinoids as a general cancer preventative on a massive scale in a general population. In addition, retinoids might also be used in conjunction with the regular chemotherapeutic cancer drugs which are given to cancer patients. Ways to measure retinol binding protein are needed. Concentrations of retinol binding protein and its relationship to various disease states, particularly cancer, should be determined.

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VITAMIN D - ITS EXCESSIVE USE IN THE U.S.A.

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INTRODUCTION

Sir Edward Mellanby (1920) first demonstrated that cod-liver
oil could prevent rickets. Mellanby (1920) named this antirachi-
tic material "fat soluble vitamin D." In retrospect it is tragic
that this extremely potent sterol was classified as a vitamin; its
reclassification as a potent, carefully controlled hormone should
be seriously considered (Loomis, 1967). There is a mass of pub-
lished information on the toxic effects of excesses of vitamin D
on the arterial system in man (Forfar et al., 1956; Taussig,
1966; Seelig, 1969; Taylor et al., 1972 and Scientific reviews
(GRAS) food ingredients - vitamin D, NTIS, U.S. Department of
Commerce, 1974) and animals (Hass et al., 1958; Bajwa et al.,
1971; Scientific reviews (GRAS) food ingredients - vitamin D,
NTIS, U.S. Department of Commerce, 1974; Liu et al., 1979 and Peng
et al., 1978).

The saga of the use and abuse of vitamin D since the first
demonstration of its antirachitic properties in 1920 is most in-
teresting. It appears that a combination of an initial lack of
understanding of the actions and metabolism of vitamin D plus a
general expectation that vitamin D would have a favorable thera-
peutic effect on many skeletal diseases led to a rather serious,
extensive and somewhat protracted overuse of this agent. The
report by Seelig (1969) covers the history of our development of
knowledge of vitamin D very thoroughly.

REVIEW OF PERTINENT LITERATURE

History of Development of Knowledge of Vitamin D Requirements

It was observed in 1953 and 1954 that approximately 100 new
cases of infantile hypercalcemia were being diagnosed annually in
Great Britain. This observation led to a survey of the vitamin D
enrichment practices in Great Britain (Lightwood et al., 1956;
Lightwood et al., 1957 and Forfar, 1956). This survey disclosed
that the then liberal enrichment of national dried milk, enrichment
of infant cereals plus the daily ingestion of one teaspoonful of
national cod-liver oil compound containing 700-800 I.U. would
result in an ingestion of about 4,000 I.U. of vitamin D per day.
Interestingly, a study in Glasgow (Graham, 1959) revealed that 26
infants and children had hypercalcemia while ingesting about 2,000
I.U. per day and 8 receiving about 1,000 I.U. of vitamin D per day
showed hypercalcemia. It should also be pointed out that since
essentially all British infants were ingesting 3,000 to 4,000 I.U.
of vitamin D per day there were many individuals who suffered no
ill effects. These studies suggested a rather wide range of
tolerance to ingestion of variable excessive amounts of vitamin D.
Following this study vitamin D was removed from infant cereal and
from national dried milk; the amount of vitamin D in national cod-
liver oil was also reduced significantly. Since daily intake of
vitamin D in Great Britain has been reduced to about 400 I.U. the
occurrence of hypercalcemia in infants and children has been
essentially eliminated. Jeans (1950) reported a recommended daily
intake of 400 I.U. of vitamin D (for newborns, infants, growing
children to 19 years of age and pregnant and lactating females)
which is generally accepted in the Western World.

More recently the Committee on Nutrition, American Academy of
Pediatrics has published an excellent study on the prophylactic
requirements and the toxicity of vitamin D (Fomon, 1963). This
committee wisely recommends minimal but adequate daily intakes of
vitamin D. A daily intake of 400 I.U. of vitamin D for premature
and term infants, children and adolescents up to age 19 is recom-
mended. No vitamin D is recommended for adult males and non-preg-
nant adult females. The committee further advised that pregnant
females during the second half of gestation and lactating females
should receive 400 I.U. of vitamin D. It should be mentioned that
Loomis (1967) states that unpigmented skin of man, when exposed
to solar radiation, converts a significant amount of 7-dehydro-
cholesterol to vitamin D_3; he indicated that about 20 to 25 square
centimeters of minimally pigmented skin (an area of skin present
on one cheek or the back of one hand) when exposed to 3 hours of
sunshine can convert a sufficient quantity of 7-dehydrocholesterol
to 400 I.U. of vitamin D_3. Individuals with more pigment in their
skin such as Blacks and Indians have a reduced capacity to synthe-
size vitamin D in skin exposed to sunlight. The author states that

persons with more pigment in their skin, such as Blacks and Indians, who live in inland areas far from the equator have a greater propensity to develop rickets if they have no oral or subcutaneous sources of vitamin D. Persons with pigmented skin living on the sea coast (ex. Eskimos) are not susceptible to rickets because of the rich source of vitamin D in fish liver and other tissues of fish.

Vitamin D Concentration in Foods

Fomon et al. (1963) include a table in their paper (Table IIA, page 519) which lists amounts of vitamin D added to various foods. They list 25 common food products eaten frequently) which contain very significant quantities of vitamin D (80 to 1,000 I.U. vitamin D per serving). During the past 16 years the addition of vitamin D to frequently ingested foods has become much more common. Kummerow (1979) presents data indicating that the average daily per capita intake of vitamin D from vitamin D enriched foods is 2435 I.U. If an individual is also taking one vitamin capsule containing 400 I.U. of vitamin D each day his daily vitamin D intake is 2835 I.U.; this level of daily vitamin D dosage is comparable to the toxic levels of vitamin D intakes reported by the British in 1953 and 1954. (Lightwood et al., 1956; Lightwood et al., 1957 and Forfar et al., 1956).

Another important source of dietary vitamin D is that present in meats and fish. Kummerow et al. (1976) and Kummerow (1979) present startling data on the excess amounts of vitamin D added to animal feeds with a resultant retention of vitamin D in various edible portions of these animals (Examples:- when layer chickens were fed a commercial ration which assayed at 1,600 I.U. of vitamin D per pound their skin contained 4,086 I.U. vitamin D/pound, breast muscle - 363 I.U. and liver 1,725 I.U. of vitamin D. When swine were fed 100,000 I.U. of vitamin D/pound of commercial ration for 6 weeks, their lean muscle contained 5,765 I.U. and liver 2,270 I.U. of vitamin D per pound). Fish frequently have very rich concentrations of vitamin D which are not of exogenous origin; their vitamin D is endogenously synthesized (Example: vitamin D content of rock fish - 2,700 I.U. of vitamin D per pound). Kummerow (1979) states that the average per capita intake from vitamin D enriched foods is 2,435 I.U. which is six times the National Research Council requirements. Further, many persons take an additional 400 I.U. of vitamin D in a daily vitamin capsule.

Absorption, Turnover and Storage of Vitamin D

The metabolism and turnover of vitamin D is important and should be discussed (Avioli et al., 1967) as well as the accumula-

tion of vitamin D in tissues resulting from excess vitamin D intake (Kummerow et al., 1976). Avioli et al. (1967) report that vitamin D excretion is principally via the enterohepatic circulation; its excretion is comparable to that of cholesterol. The biological half-life of labeled vitamin D_3 is nearly 5 days (112.5 hours). Since vitamin D is a fat soluble vitamin, once it gains entrance to the organism (via injection or intestinal absorption) its elimination is a slow process. If given in excess vitamin D cannot be excreted rapidly by the kidneys like the water soluble vitamins. Excess intake of vitamin D leads to profound increases in its content in tissues and serum; Kummerow et al. (1976) have shown that feeding 100,000 I.U. of vitamin D_3 per pound of ration to weanling pigs for 6 weeks results in a more than 15 fold increase in vitamin D_3 content in skeletal muscle and a 12 fold increase in serum vitamin D_3 concentration. They also present data showing a 40 fold increase in skeletal muscle of vitamin D_3 content of humans given massive doses of vitamin D_3 for osteomalacia. They reported no data on accumulation of vitamin D_3 in arteries, kidney or heart. These tissues are most sensitive to excessive accumulation of vitamin D and will be discussed in the next section.

Toxicity of Excess Vitamin D

     Excessive intake of vitamin D can adversely affect a number of tissues (Seelig, 1969; Hass et al., 1958 and Liu et al., 1979). The heart and kidneys have an intermediate sensitivity to vitamin D. When there is an excess intake of vitamin D, morphologic and functional changes involving the heart and kidneys become apparent rather early (Seelig, 1969; Liu et al., 1979); both organs show abnormal accumulation of calcium which destroys functional cardiac and renal tissue. Liu et al. (1979) observed severe diffuse calcific cardiomyopathy with cardiac failure after 23 weeks of excess vitamin D intake plus nicotinism in rhesus monkeys. Seelig (1969) presents strong evidence that excess intake of vitamin D is the cause of the supravalvular aortic stenosis syndrome; she also presents evidence of renal damage due to hypervitaminosis D.

     There is an abundance of experimental evidence in many species of animals indicating that arterial tissues are most susceptible to hypervitaminosis D. (Hass et al., 1958; Bajwa et al., 1971; Scientific Reviews (GRAS) food ingredients-vitamin D, NTIS, U.S. Department of Commerce, 1974; Liu et al., 1979 and Peng et al., 1978). Evidence implicating mild, protracted hypervitaminosis D in human arteriosclerosis is also quite convincing (Forfar et al., 1956; Taussig, 1966; Meyer & Stelzig, 1968; Seelig, 1969 and Taylor et al., 1972). The arteries' elastic tissue is the principal target tissue of excess vitamin D; elastic membranes and adjacent ground substance accumulate calcium and are severely damaged or

disrupted. Smooth muscle cells are less susceptible but at a later
stage undergo necrosis (Hass et al., 1958; Hass et al., 1960 and
Liu et al., 1979). Peng et al. (1978) reported a very significant
study in which minimal daily excesses of vitamin D_3 produced ar-
terial damage in squirrel monkeys. The established daily vitamin
D_3 requirement for squirrel monkeys is 100 I.U. (Lehner et al.,
1967); it is of interest that vitamin D_2 is essentially inactive in
squirrel monkeys. Daily ingestion of 500 I.U. of vitamin D_3 plus
0.5 percent cholesterol in the diet produced early but significant
arteriosclerosis within 10 months (Peng et al., 1978). Daily in-
takes of 1,000 I.U. of vitamin D_3 without the addition of dietary
cholesterol for 10 to 18 months also produced severe arteriosclero-
tic changes (Peng et al., 1978).

When one reflects upon the fact that the average person in the
U.S. ingests 6 to 7 times the maximal, recommended daily amount,
400 I.U. vitamin D (for infants, children and pregnant females -
adult males and non-pregnant females require no exogenous vitamin
D) over many decades, it is shocking to contemplate the effect of
ingestion of 5 to 10 times the daily required dose of vitamin D on
the arteries of squirrel monkeys. If significant arterial damage
and repair can be induced in squirrel monkeys by feeding 5 to 10
times the recommended daily intake for 10 to 18 months it is
frightening to contemplate what the daily ingestion of 6 to 7 times
the required daily intake of vitamin D (for infants and pregnant
women - 400 I.U.) might do to the arterial tree of humans over a
period of 50 or more years. Consideration of the above information
suggests that "operation over-kill" in the area of vitamin D in-
gestion in this country may be the most significant risk factor in
the genesis of arteriosclerosis; cholesterol intake and levels of
serum cholesterol may well be only important secondary factors in
the pathogenesis of atherosclerosis.

It should be re-stated that vitamin D is a fat soluble vitamin.
After it has been absorbed or ingested its elimination is a slow
process principally via the enterohepatic circulation. All persons
interested in nutrition as related to cardiovascular disease should
always be mindful that vitamin D is not a water soluble vitamin and
that excess intakes of this vitamin cannot be rapidly excreted
through the kidneys.

Finally, it should be mentioned that the traditional "end-
stage" complication of human arteriosclerosis, arterial thrombosis,
has been produced in rabbits (Hass et al., 1960) and monkeys (Liu
et al., 1979) by administration of vitamin D, nicotine and choles-
terol to these animals.

SUMMARY AND CONCLUSIONS

Vitamin D was first identified in 1920. Numerous clinical and experimental studies have been reported. In 1950 a recommended daily intake of 400 I.U. for premature and term infants, children and adolescents up to 19 years and pregnant and lactating females was reported and accepted; no exogenous vitamin D was recommended for adult males and non-pregnant females.

Many processors of foods add vitamin D to a wide variety of foods. It has been estimated that the average person in the United States ingests 2,435 I.U. of vitamin daily which is added to many foods; also, many persons take 400 I.U. of vitamin D in a daily vitamin capsule. There is also a great propensity for those feeding hogs, chickens and beef to add massive quantities of vitamin D to animal feeds. This results in accumulation of shocking amounts of vitamin D in meat products consumed by persons in this country. Data was presented indicating that the average person on a conventional diet consumes more than 6 to 7 times the recommended daily intake of 400 I.U. of vitamin D. The excessive consumption of vitamin D represents another example of "operation over-kill" in the U.S.A. It seems that those making policies for vitamin D intake in this country are not remembering that vitamin D is a fat soluble vitamin with a very slow excretion rate. When excess vitamin D is absorbed into the body it cannot be excreted rapidly by the kidneys like the water soluble vitamins. The half turnover time is about five days. Excess amounts of vitamin D are stored in various tissues, e.g. cardiac muscle, skeletal muscle, kidneys and arterial walls.

It has been shown that a daily intake of five to ten times the required dose of vitamin D can produce significant arteriosclerosis in squirrel monkeys within ten months. It seems likely that man's daily intake, which is seven times greater than that required for a growing child, may well be the major cause of his arteriosclerosis; the average adult in this country may be taking this excessive vitamin D for 50 to 60 years. Cholesterol intake and elevated serum cholesterol levels are probably secondary contributing factors in the development of arteriosclerosis.

It is suggested that all individuals involved in the planning, production and distribution of food and vitamins in the United States develop and enlightened consciousness regarding vitamin D intake and the grave complications related to prolonged, moderate overdosage of this vitamin. It is also suggested that all persons in this country check labels on their food packages for their vitamin D content in order to avoid chronic hypervitaminosis D.

Nutritionists should seriously consider establishing a national committee to evaluate vitamin D additives in foods; hopefully, the

rather promiscuous habit of adding significant amounts of vitamin D
to many foods could be corrected. The Food and Drug Administration
might also increase its involvement in this area in order to make
certain that many individuals in the U.S. are not unknowingly in-
ducing mild, chronic hypervitaminosis D by daily ingestion of excess
vitamin D.

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THE CLINICAL IMPLICATIONS OF LIPID

ANTIOXIDANT NUTRITION

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BACKGROUND

Few areas in human nutrition are so charged with claims and counterclaims as is that of the antioxidant nutrients, and vitamin E specifically. The very name of vitamin E, tocopherol, reflects the considerable controversy which surrounds it, in that it is derived from the Greek word meaning "bringing forth in childbirth." The fertility effect, however, since its early demonstration in rats by Evans and Bishop (1923), has not been demonstrated in humans. Vitamin E was discovered when Evans and Bishop (1923) isolated from vegetable oils a substance that helped rats produce robust offspring. Unfortunately, this observation was picked up by many individuals and promoted in the lay literature as a human "sex" vitamin. This clouded the scene with regard to the biological importance of vitamin E and has led to much misunderstanding concerning both the prophylactic and potential therapeutic benefit of vitamin E and other members of the lipid antioxidant family for the human being. The controversy surrounding it is summed up in a statement made by Tappel (1973): "While few compelling uses have been found for vitamin E, the more research which is done on this substance, the more intriguing it appears. Thus, there is a nagging suspicion that there is a very important use for the vitamin and we are just not smart enough to see it."

In this review the biological effects of vitamin E and others of the lipid antioxidant family will be explored in hopes of better differentiating between the supportable or therapeutic uses and those which are based solely upon testimony.

The early history of vitamin E in the period of 1922 to 1932 was fraught with considerable controversy as to what it was and how it actually played a significant role in animal physiology (Mason, 1977). Sterility in rats was found in high-protein, low-fat diets by Osborne, Mendel and Mattill. Evans and Bishop proposed the presence of an antisterility factor in 1922, and fetal death and resorption in rats as a result of deficiency of this factor was reported by Mattill in 1924. Testicular degeneration was then found in the animals treated with a fat-deficient diet, and the essence of this substance termed vitamin E, as being related to these fertility changes, was propounded in the monograph by Evans and Burr, entitled "The Antisterility Vitamin: Fat-soluble E" in 1927. It was not, however, until 1931 that Olcott and Mattill recognized the potential antioxidant effect of vitamin E.

In 1936, Evans et al. isolated a pure fraction named alpha-tocopherol, and in 1937 Emerson et al. isolated the beta and gamma tocopherol forms while in that same year Olcott and Emerson discussed the mechanism of action of vitamin E, utilizing an anti-oxidant model. The structural formula of alpha-tocopherol was published by Fernholz in 1938, as shown in Figure 1. During this period it was found that vitamin E deficiency produced a nutritionally-induced muscular dystrophy in rabbits, similar to forms which had been observed in the guinea pig some seven years earlier. Later it was found that the muscle wasting in chicks, termed "exudative diathesis" was also related to vitamin E deficiency.

Dam's laboratory provided evidence of the antiencephalo-malasic property of alpha-tocopherol in chicks (1938), and, MacKenzie et al. (1940) found that a lack of alpha-tocopherol alone was responsible for the myopathic changes previously described in the rabbit. The two decades following 1920 witnessed the isolation of a fat-soluble constituent of food of importance in optimizing physiologic function in many animal species, but which had not yet been identified with any human deficiency disease. A major question which remained from this period was, and still is, whether the tocopherols as a family have functions other than those as biologic antioxidants in animals. It has become clear, however, that biologic antioxidation is a major pathway by which the tocopherol family and other lipid soluble antioxidants do, in fact, elicit their function.

We now recognize many human pathologic conditions as being associated with increased oxidation, particularly of the free-radical oxidation type (Dormandy, 1978). Generally speaking, free-radical oxidation is a result of an oxidant material interacting with a biological component, such as a cell membrane lipid or a protein, to produce a free-radical chain-carrying species which
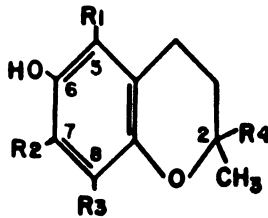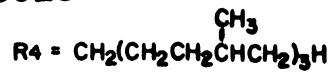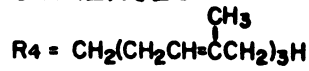
Figure 1. Chemical structures of naturally occurring tocopherols

$$A - B \xrightarrow{\Delta \text{ or } h\nu} A\cdot + B\cdot \qquad (1)$$

$$M^+ + ROOH \longrightarrow M^{+2} + \cdot OR + {}^-OH \quad (2)$$

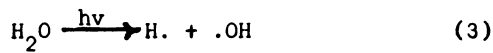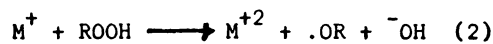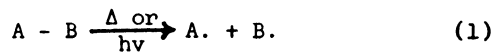$$H_2O \xrightarrow{h\nu} H\cdot + \cdot OH \qquad\qquad (3)$$

Figure 2. Generation of free radicals in biological systems by
(1) thermal or photolytic homolysis, (2) one electron
redox reactions, (3) high energy radiation and photo-
lysis of water.

can then produce considerable cell or tissue degeneration. Lipid
peroxides, for instance, are formed by a series of oxidation
reactions of unsaturated lipid material and have been identified
as important degradative biomolecules involved in the cellular
aging process. In oxygen toxicity, air pollution, oxidative damage
to cells, and a host of other degenerative biochemical transforma-
tions, the formation of lipid peroxides involves a reaction of
biochemical oxidants with polyunsaturated lipids (Bland, 1976), as
is diagrammed in Figure 2.

VITAMIN E AS A FREE-RADICAL TRAP

The free-radical intermediates, as shown in Figure 2, occur in almost all biologic systems. Cells and tissues are generally protected against oxidizing free radicals by complexing of anti-oxidant mechanisms, some of which are intimately involved with the fat-soluble vitamin families, such as the tocopherols.

In disease, these mechanisms may fail to protect or conversely the mechanisms may fail and cause disease. The primary products of free-radical oxidation undergo rapid and spontaneous fragmentation. Many of these fragments are also highly active in biologic systems. Some have considerable survival time and may induce long-term degenerative effects. In the excellent review by Dormandy (1978), he discusses the importance of free-radical oxidation and antioxidants in human health and disease and says: "Because of the numerousness and instability of free-radical oxidation products, their characterization and isolation are fraught with difficulties, but there is no longer any doubt that many are extraordinarily powerful and have an astonishing range of effects upon biologic systems. Some are cytotoxic, some have striking bacteriostatic properties, some affect platelet aggregation, some have prostaglandin-like activity on neuromuscular transmission, and some of the processes which might be influenced or governed by these products in vivo have considerable survival value." The insidious character of these free-radical-induced lipid peroxidations suggests that the mechanism of formation involves a chain-carrying propagation process as seen in Figure 3 (Bland, 1976). Conceivably, then, only a few free radicals need necessarily be produced within a biochemical system to induce considerable lipid peroxidation and associated damage.
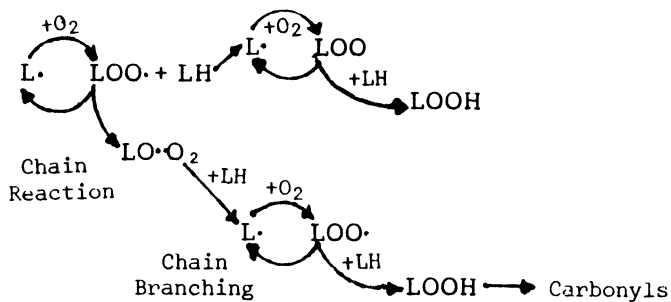

Figure 3. Mechanism of free radical reaction leading to fatty acid hydroperoxides.

Recently, Cohen and Cedarbaum (1979) have demonstrated that reduction of oxygen during microsomal electron-transfer reactions can ultimately produce hydroxyl radicals, which can participate as active chain-initiating species in production of the peroxides. Other free-radical initiators found in biological systems include hydrogen peroxide, superoxide, anion radical, and singlet oxygen. Formation of lipid peroxides is most commonly associated with regions of the biochemical systems which are abundant in polyunsaturated fatty acid phospholipids. Cellular membranes, including mitochondrial and microsomal membranes, are composed of fatty acids with two, four, five, and six double bonds, which have been shown to be rapidly peroxidized via free-radical mechanisms with increasing ease in the absence of protection by the nutrient antioxidant agents. The autooxidation of fats is a common phenomenon. In food stuffs it is known as the process which causes rancidity. It has also been shown to occur in vitro in adipose tissue and in vivo in human red blood cells under conditions of hyperoxia. It is enhanced in the presence of iron, a potent generator of free radicals, and of ascorbic acid. Co-oxidation of unsaturated fatty acids and ascorbic acid occurs as a coupled reaction with ferrous iron. The forms of vitamin E, such as alpha-tocopherol, are methyl-substituted phenolic derivatives, which have a chroman ring, accounting for their reactivity with free radicals, and a fatty acid side chain making them soluble in lipids (Figure 1).

Because of its capacity to trap free radicals, as a one-electron reducing agent, vitamin E prevents the oxidation reactions described above. As can be seen from Figure 4, as alpha tocopherol serves as a selective oxidant trap (thereby reducing the free-radical chain-carrying process), it leads to the ultimate oxidation product tocoquinone. Evidence is accumulating to demonstrate that vitamin E presents itself nicely in vivo as a selective lipid antioxidant in fat-rich organelles, such as cellular membranes. For instance, patients afflicted with beta-thalassemia major show virtually every kind of erythrocyte membrane deformation abnormality that has been described. In addition, Stocks, et al. (1972) have found an increasing susceptibility of thalassemic red blood cells to oxidant stress. Rachmilewitz et al. (1976) have found that beta-thalassemics show a nearly two-fold increase in total red cell lipids and a correspondingly larger amount of lipid per cell, which is susceptible to peroxidation. After oxidant stress they find increases in malonaldehyde concentrations which can only exist as a result of increased lipid peroxidation (Trostler et al. 1979). Graziano et al. (1976) have found recently that the iron-chelating drug 2,3-dihydroxybenzoic acid is an effective inhibitor of red cell membrane peroxidation in thalassemics. Hemolysis is almost always found to be preceded by lipid peroxidation. Barker and Brenn (1975) have found that peroxidation of red cell membrane of vitamin E deficient rats proceeds in a similar manner to the peroxidation of mycellar phospholipids in vitro, and they suggest that

peroxidation exposes both polar and nonpolar lipid sites in the red cell membrane, resulting in its weakening. Kahane and Rachemilewitz (1976) have recently found that alterations due to increased lipid peroxidation in thalassemics' erythrocyte membranes can be prevented by the ingestion of larger amounts of vitamin E, which is consistent with the antioxidant model for the tocopherols.

Recently Bowie et al. (1979) have reported that the crisis of sickle cell anemia is associated with increased lipid peroxidation of the red cell membrane leading to increased malonaldehyde concentrations of membrane lipids. The sickle cells are much more susceptible to oxidatively-induced hemolysis when exposed to hydrogen peroxide than normal red cells. Vitamin E, when present in the incubation medium, was found to suppress the formation of malonaldehyde in the sickled red cell when exposed to peroxide. Interestingly, intracellular calcium, which is known to become elevated during the formation of irreversibly sickled cells, also was decreased at high vitamin E levels.
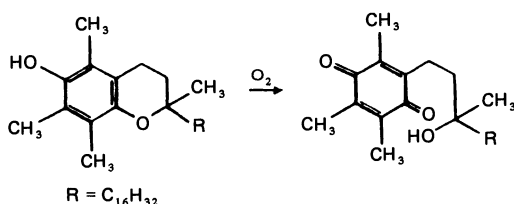

Figure 4. Oxidation of α-tocopherol

Vitamin E does not participate in this process of antioxidation by itself. It works in conjunction with other nutrient-derived antioxidants, as can be seen in Figure 5. The interaction of vitamin E with the selenium-activated enzyme, glutathione peroxidase, and sulfur amino acids renders the lipid-rich regions of tissue systems much less susceptible to peroxidative damage induced by free radical reactions.

VITAMIN E DEFICIENCY AND ANEMIAS

Vitamin E deficiency in infants is known to lead to hemolytic anemia, with the typical syndrome appearing at four to six weeks of
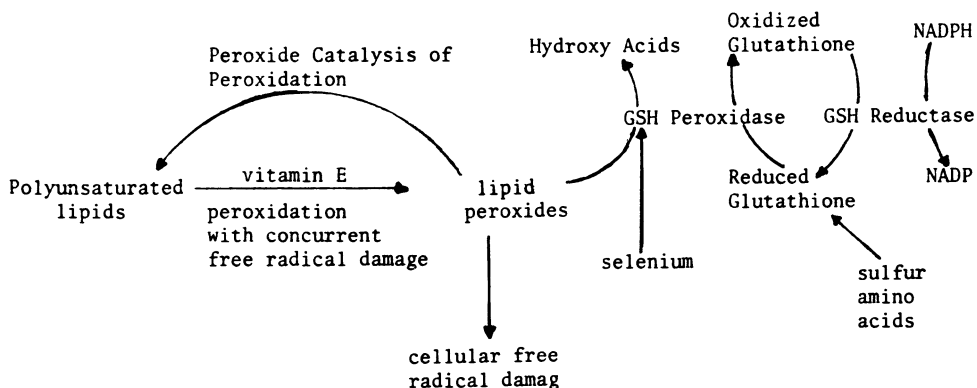
Figure 5.   Interactions among selenium, vitamin E, and sulfur
            amino acids in inhibition of lipid peroxidation
            damage.

age in infants of birth weights less than 1500 grams, and is
characterized by reticulocytosis, thrombocytosis and low hemoglobin
values.   Blood studies have revealed a variable red cell morphology,
with decreased plasma concentrations of vitamin E and very marked
increased sensitivity of the erythrocytes to hemolysis induced by
exposure to dilute solutions of hydrogen peroxide.   This demonstrates
that these infants are unable to withstand oxidative stress and
thereby have increased tendencies toward lipid peroxidation with
the attendant loss of membrane integrity and function, resulting in
anemia.   A number of factors have been found to contribute to the
vitamin E deficient status of the small premature infant.   Even full-
term infants with birth weights of 3500 grams or more are relatively
vitamin E deficient at birth, having plasma concentration of 0.2 to
0.6 mg/dl (normal adult 0.75 to 0.92 mg/dl), and body stores of
about 20 mg.   Breast-fed infants quickly achieve adult values, but
infants fed artificial formula may take months to reach adult values
if their diet is not adequately supplemented.   It is interesting to
note that iron supplements, which are commonly given to these low-
birth-weight babies, increase the rate of free-radical peroxidation,
and can actually exacerbate this vitamin E deficiency in premature
infants (Oski and Barness, 1967).   The studies of Melhorn and Gross

(1969) demonstrated that administration of exogenous iron at a dose
of 8 mg/kg/day to vitamin E deficient premature infants produced a
more severe anemia than non-iron supplementation. Their conclusion
was that iron precipitated hemolysis in the vitamin E deficient state
by increasing free radical peroxidation. The work of Oski (1977) has
demonstrated that these premature infants, when given iron in con-
junction with d-alpha tocopherol administered intramuscularly in a
dose of 200 to 800 mg, responded by a rise in hemoglobin from 7.6
to 9.8 g/dl, and a reduction in the hydrogen peroxide hemolysis
test from 80 percent to 8 percent. In a subsequent study it was
shown that daily administration of 25 mg of vitamin E by mouth,
starting in the first week of life will prevent the hemolytic anemia
associated with vitamin E deficiency in the low-birth-weight
premature infant. The optimum dose was found to be 125 mg/kg of
dl-alpha-tocopherol in a water dispersible vehicle, administered
intramuscularly in four divided doses on days one, two, seven and
eight of life.

The antioxidant action of vitamin E may have important
implications for the integrity of other cells in addition to the red
cells that are susceptible to oxidant-induced injury. Of particular
importance to the human infant is a possible role of vitamin E
deficiency in the genesis of retrolental fibroplasia and broncho-
pulmonary dysplasia. Johnson and associates (1976) have shown that
even when oxygen is carefully administered, controlled or omitted,
retrolental fibroplasia may occur in premature infants because the
retinal vessels, which usually develop during intrauterine life at
oxygen tensions of 50 to 55mm/Hg, are exposed to abnormally high
tensions before retinal vascularization is complete. The result
may be vasoconstriction and obliteration of retinal arteriols and
capillaries. Johnson et al. (1976) conducted a clinical trial in
which alternate infants with birth weights of less than 2000 g were
started on vitamin E or a placebo within four to 24 hours of birth.
The study was designed to determine if the vitamin E supplemented
infants who had higher serum levels of vitamin E had less incidence
of retrolental fibroplasia, or regression of the symptoms as
determined by indirect ophthalmoscopy. Follow-up of 48 infants at
one year of age revealed that six of eight with retrolental fibro-
plasia treated with vitamin E had normalized as compared with seven
of 18 who received placebo injections, and one of 12 who were not
treated.

Recently, Ehrenkranz et al. (1978) have reported that
respiratory distress syndrome in neonates who develop broncho-
pulmonary dysplasia can be significantly ameliorated by the adminis-
tration of vitamin E. Twenty infants received vitamin E administered
intramuscularly during the acute phase of the syndrome and twenty
infants served as controls. Administration of vitamin E signifi-
cantly increased the serum vitamin E concentration. None of the nine

vitamin E treated patients had changes characteristic of broncho-
pulmonary dysplasia, and all survived, whereas six of the 13 controls
had x-ray changes consistent with the condition and four died. The
conclusion of the authors was that administation of vitamin E during
the acute phase of the respiratory distress syndrome appears to modify
significantly the development of dysplasia.

     Consistent with this view that infants, and more specifically
premature infants of low birth weight, are susceptible to increased
lipid peroxidation as a result of antioxidant insufficiencies is a
report from Money (1978). He reported the low liver contents of
vitamin E, selenium, and vitamin A in infants with sudden infant
death syndrome as compared to that of control children. The results
of this study indicate that low levels of vitamin E and organoselenium
compounds may be important in the development of certain forms of
sudden infant death syndrome and should be given further attention.
Further, ferrous sulphate, an iron supplement which is known to in-
crease peroxidation, could involve a special risk.

VITAMIN E INSUFFICIENCY IN THE ADULT

     One of the lengthiest and most extensive of the studies in
adults with regard to vitamin E nutriture was that of Horwitt and
coworkers (1960, 1962, 1978). Under the sponsorship of the Food
and Nutrition Board of the National Academy of Sciences, this long-
term project was begun in 1953 to determine the need for vitamin E
by adult male subjects. Low plasma tocopherol levels and increased
susceptibility of red blood cells to hemolysis in the presence of
hydrogen peroxide were used as criteria of insufficient vitamin E.
The basal diet supplied about three milligrams of d-alpha-tocopherol
daily, and depletion occurred slowly, but was found to be acceler-
ated by increasing the daily consumption of polyunsaturated fatty
acids, principally linoleic acid. This is presumably a result of
the increased need of antioxidants to protect the reactive sites of
unsaturation in these fatty acids against peroxidation. During the
course of this eight-year study, several changes were made, each
one accentuating the decrease in plasma tocopherol. Horwitt (1956)
observed that subjects maintained on low tocopherol diets for long
periods of time showed small, but significant, decreases in
erythrocyte lifetime and that subsequent administration of 300 mg
d-alpha tocopheryl acetate daily resulted in small, but significant,
increases in reticulocyte count. These results were consistent with
those of Marvin et al. (1960), who showed a substantial decrease in
erythrocyte survival time in monkeys which were severely deficient
in vitamin E.

     Malabsorption syndromes were long suspected as causes of
vitamin E deficiency. In some cases of cystic fibrosis, chronic
pancreatitis, biliary atresia, and celiac disease, findings of

extensive deposition of lipid peroxidized material, such as ceroid,
and muscle necrosis were taken as indirect evidence of vitamin E
deficiency (Herting, 1966). Increasing numbers of reports have
demonstrated the direct correlation between the classic signs of
vitamin E deficiency (including creatinuria, ceroid deposition,
muscle weakness, increased serum creatine phosphokinase, and sub-
normal levels of alpha tocopherol in blood or adipose tissue) in
cases of cystic fibrosis, biliary atresia, nontropical sprue,
xanthomatous biliary cirrhosis, chronic pancreatitis, and a host
of other conditions generally characterized by steatorrhea. Thus,
there appears to be little question that malabsorption syndromes
lead to vitamin E deficiencies in the human.

In a recent report by Tsai et al. (1975) the effect of mega-
vitamin E supplementation in humans was examined. Two hundred and
two subjects were randomly assigned to treatment groups, one
receiving 600 I.U. of dl-alpha tocopheryl acetate daily and the
other a placebo tablet. The experiment was double blind and pro-
ceeded for a period of four weeks. Results of this study showed
that the megavitamin E supplementation did not have an effect on
work performance, sexuality, and general well being, and did not
cause muscular weakness or gastrointestinal disturbances, based
upon subjective evaluations of the treated group. Vitamin E
supplementation at this level did not affect the prothrombin time,
total blood leucocyte count, or serum creatine phosphokinase
activity of the subjects. Serum triglyceride levels were signifi-
cantly elevated in vitamin E supplemented females, but not in males.
Megavitamin E supplementation significantly decreased serum tri-
and tetra-iodothyronine concentrations in males and in females not
using steroid oral contraceptive agents. In general, then, the
study indicated that under experimental conditions of the study,
vitamin E supplementation at fairly large levels does not have
significant beneficial or undesirable effects on general health
conditions.

Recently, evidence has accumulated which suggests that higher
doses of vitamin E than the National Academy of Sciences Recommended
Dietary Allowance of 15 I.U. per day may be advantageous in certain
circumstances in promoting enhanced antioxidant activity when an
individual is exposed to oxidant stress. Working with human red
blood cells, our group found that when 600 I.U. of vitamin E are
administered orally on a daily basis an enhanced protection against
photodynamic-induced oxidative damage to the erythrocyte (achieved
by exposing the red blood cell to oxygen and light simultaneously)
can be achieved in vivo as compared to the same group serving as
its own control on a non-vitamin E supplemented regime (Bland et al.
1975). Figure 6 shows the significant difference between the
vitamin E supplemented group and the same group with no vitamin E
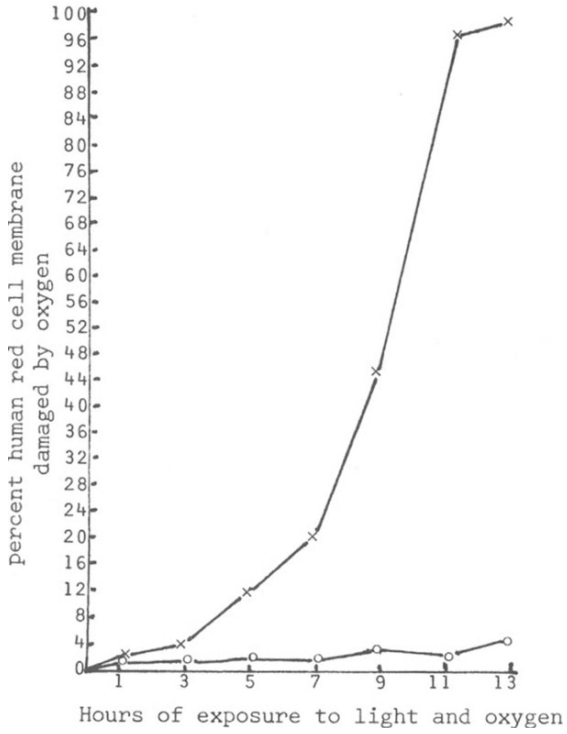supplementation. This work in vivo is consistent with the work of

Figure 6.   Effect of α-tocopherol on human red blood cell
            sensitivity to photo-induced membrane damage.
            x – controls, ingesting normal diet unsupplemented
            O – same subjects, ingesting normal diet supplemented
                with 600 IU α-tocopherol daily
            from Bland et al. (1975).

Goldstein and Harber (1972), which demonstrated that alpha-
tocopherol was found to be an effective agent in providing partial
protection against photohemolysis when erythrocytes from erythro-
poietic protoporphyric donors were treated in vitro with an emulsion
of vitamin E and then exposed to long wave-length ultraviolet
radiation in the presence of oxygen. As was demonstrated by Grams
et al. (1972), vitamin E is readily oxidized in the presence of
light, oxygen and a sensitizing dye, such as hemoglobin. The model
which has been formulated to account for the ability of vitamin E
to prevent oxidative hemolysis of red blood cells involves the
inhibition of conversion via photooxygenation of membrane-bound
cholesterol to its hydroperoxide (as shown in Figure 7), or possibly
other unsaturated fatty acids to their hydroperoxides, which could
then participate in free radical chain-carrying destruction of the
cell membrane. Our work has shown that vitamin E treated donors have
red cell membranes with higher vitamin E concentration and an
enhanced ability to prevent the conversion of membrane-bound
cholesterol to its hydroperoxide. This hydroperoxide leads to
membrane deformation and increased membrane lysis (Lamola et al.
1973). Incorporation of cholesterol hydroperoxide into the erythro-
cyte membrane has been demonstrated by Lamola et al. (1973) to
create a much more fragile erythrocyte membrane, as shown in Figure 8.
The erythrocyte system is uniquely disposed to oxidative hemolysis,
in that the hemoglobin in the red cell is a very efficient photo-
sensitizer of oxygen to its excited species, singlet oxygen, which
can then participate vigorously in peroxidation of unsaturated
residues within the cell membrane bilayer. In fact, Lubin et al.
(1972) report evidence for involvement of hemoglobin-bound oxygen
in hemolysis of vitamin E deficient erythrocytes. To determine the
active oxidant species in the erythrocyte of the vitamin E insuf-
ficient case, we examined the appearance of cholesterol hydro-
peroxide in the erythrocyte membrane, utilizing a C_{14} cholesterol-
labeled membrane in the presence and absence of various levels of
vitamin E. In Figure 9, it can be seen that in the absence of
vitamin E, the rate of production of hydroperoxide within the
cellular membrane was considerably greater than in the cells which
had been treated with 0.56 mM tocopherol (Bland et al. 1978).
Merkel and Kearns (1972) have shown that one of the potential
oxidant species in this process, singlet oxygen (an activated
oxidation species some 22 Kcals per mole above the ground state
energy of oxygen), is intimated to be involved in many of these
photodynamic, light-induced oxidation processes, and has a ten-fold
longer lifetime in deuterium oxide than in normal water. This
enhanced lifetime has been used as a test for the presence of
singlet oxygen in photooxidations. As can be seen in Figure 10, the
rate of photoperoxidation is greatly enhanced in the presence of a
selective singlet oxygen trapping agent, isobenzofuran. These data
then clearly implicate the involvement of singlet oxygen in the
erythrocyte photooxidation process and demonstrate that tocopherols
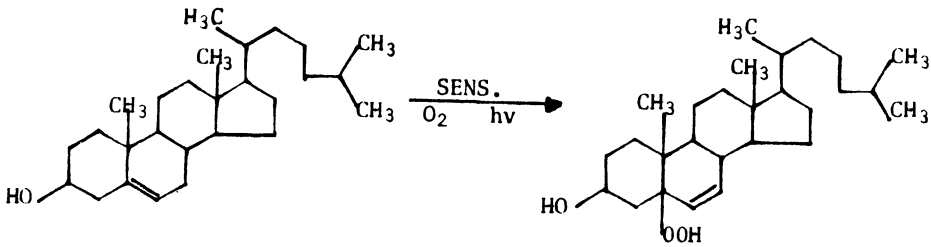
Figure 7. Photooxidation of cholesterol and conversion to its
           5-hydroperoxide.
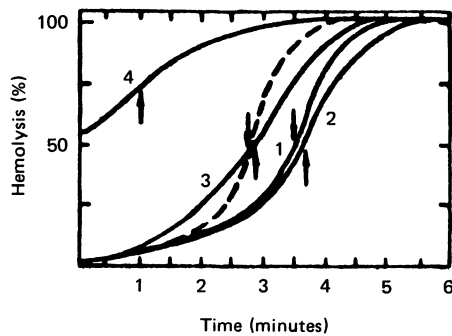

Figure 8. Cholesterol hydroperoxide effects upon the stability
           of the erythrocyte membrane. The solid curves show
           hemolysis of cholesterol hydroperoxide containing
           RBC after incubation with the hydroperoxide for 10
           minutes (curve 1), 20 minutes (curve 2), 50 minutes
           (curve 3) or 80 minutes (curve 4) at 37°C. The
           arrows represent the time taken in each for 50 percent
           hemolysis to occur.

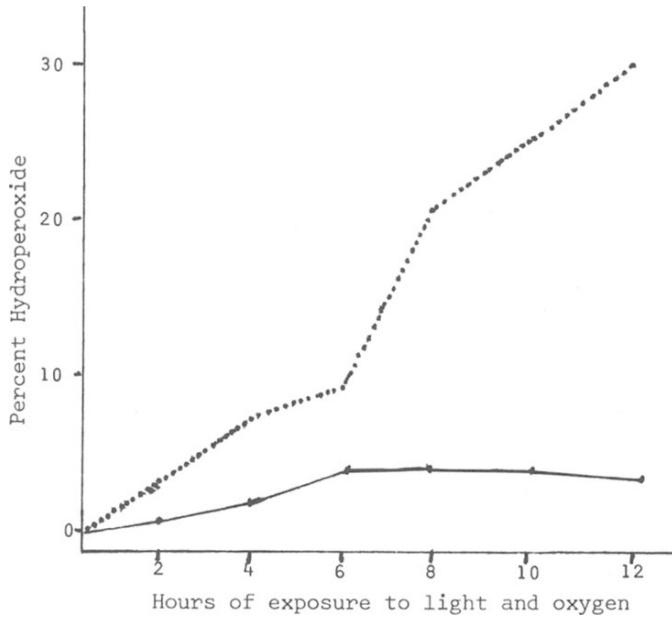Figure 9. ^{14}C-Cholesterol-labeled erythrocyte membrane
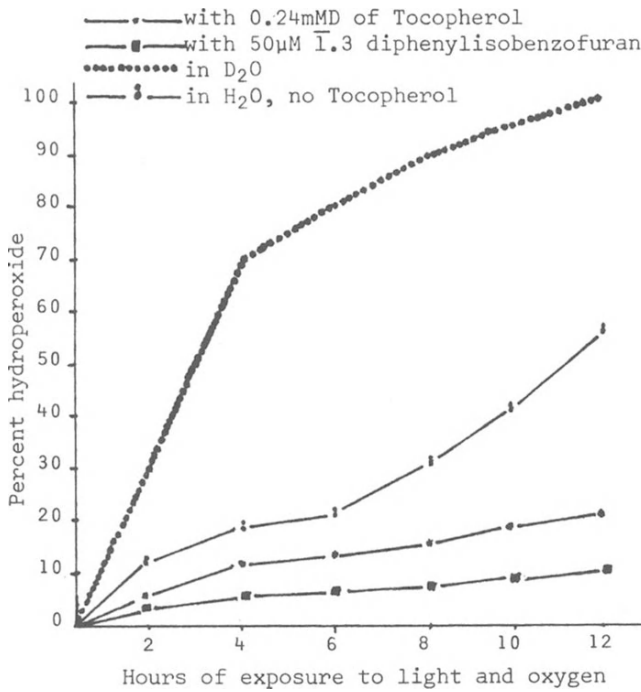   photooxidation (Bland et al. 1978).

Figure 10.   Influence of D_2O and isobenzofuran on erythrocyte
             membrane photoxidations (Bland et al. 1978).

must selectively react with singlet oxygen before the oxidant reacts
with singlet oxygen in solution to yield a variety of oxidation
products. A recent paper by Matsushita et al. (1977) has confirmed
the fact that the tocopherols have very significant quenching effects
upon photosensitized oxidation resulting from the production of
singlet oxygen. Prince and Little (1973) have found that tocopherol
reduces the radiosensitivity of mammalian erythrocytes and again
suggests that its effect upon exposure of the erythrocyte to high
energy radiation is that of protection against oxidative damage.
Other types of blood abnormalities which have been associated with
vitamin E use in humans include congenital dyserythropoietic anemia
of the type II variety and reduction of osmotic fragility in beta-
thalassemia major.

The work of Steiner (1977) has suggested that vitamin E has an
inhibitory effect upon platelet aggregation, presumably through the
inhibition of the endogenous platelet conversion of arachidonic
acid into prostaglandin. The increased platelet aggregation observed
by Machlin et al. (1975) in vitamin E deficient rats was found to be
related to increased prostaglandin synthesis. White et al. (1977)
suggest that vitamin E inhibits platelet prostaglandin synthesis by
blocking conversion of arachidonic acid into a free radical state
necessary for the insertion of arachidonic acid into the cyclo-
oxygenase or lipooxygenase pathways. This raises then the
potentiality that vitamin E may have hematological effects other than
just its antioxidant effect. The suggested role that vitamin E plays
in the synthesis of various prostaglandins in the platelet and else-
where in the body may be an important area for future research.

Recently, Meyers et al. (1977) have found that the anti-tumor
antibiotic adriamycin, which is known to induce severe cardiac
toxicity by way of lipid peroxidation, can have its cardiac
toxicity reduced by prior treatment of animals with the free radical
scavenger tocopherol. The treatment with tocopherol does not,
however, alter the magnitude or duration of the adriamycin-induced
suppression of DNA synthesis in P388 ascites tumor, nor does it
diminish the anti-tumor responsiveness of P388 ascites tumor. This
remarkable result suggests that adriamycin, and potentially other
cytotoxic agents, has at least two mechanisms of tissue damage:
one which involves lipid peroxidation, which is blocked by toco-
pherol and may result in cardiac toxicity, and one which involves
binding to DNA, is not antagonized by tocopherol and is responsible
for tumor response. It might be suggested from these data that most
all drugs which are known to induce increased free radical peri-
oxidation damage may have some of their side effects ameliorated by
pretreatment of the patient with vitamin E.

RELATIONSHIP OF VITAMIN E DIETARY LEVELS TO SERUM VITAMIN E
CONCENTRATIONS

     Farrell and Bieri (1975), in assessing the possible toxic
and/or beneficial effects of vitamin E supplementation, examined a
group of 28 adults who were voluntarily ingesting 100 to 800 I.U.
tocopherol per day for an average of three years. No evidence of
toxicity was apparent on reviewing past medical histories with the
subjects. Plasma tocopherol was found to be elevated significantly,
from 650 μg/dl (control group mean) to 1340 μg/dl (experimental
group mean). Plasma alpha tocopherol concentrations were found not
to correlate with total daily dose in this group, but did relate
to plasma triglyceride and cholesterol concentrations. Laboratory
screening for potential toxic side effects of vitamin E supple-
mentation by performance of 20 standard clinical blood tests
failed to reveal any disturbance in liver, kidney, muscle,
thyroid, erythrocytes, leucocytes, coagulation parameters, or blood
glucose concentrations. It was concluded from this study that mega-
vitamin E supplements in this group produced no apparent toxic side
effects, and that subjective claims for beneficial effects were
highly variable. Herting and Drury (1965) compared plasma tocopherol
concentrations to erythrocyte hemolysis data in normal subjects,
stating that plasma alpha-tocopherol levels less than 0.5 mg/dl
suggested vitamin E insufficiency. Average plasma tocopherol levels
were found to range between 0.358 to 0.507 mg/dl, suggesting that
there was considerable incidence of vitamin E insufficiency in the
subjects studied in Pittsburgh, Pennsylvania and New York.

     The question as to the absorption of dietary vitamin E, in that
it is a fat-soluble vitamin and would be expected to be more trans-
ported through the lymphatic system than the portal blood system
into the liver, has been examined by several investigators.
Blomstrand and Forsgren (1968) administered radio-labeled tocopheryl
acetate to human subjects whose thoracic ducts were cannulated.
Serial samples of lymph were collected and vitamin E concentration
was determined. Their results show that tocopherols were absorbed
via the lymphatic pathway in humans, that tocopherol enters the
thoracic duct lymph intact, and that tocopheryl acetate is split
and recovered as tocopherol, presumably via the action of lipase or
esterase enzymes in pancreatic juice. It was found that in patients
with biliary obstruction the lymph contained only minute quantities
of vitamin E and therefore gallbladder problems, pancreatitis, or
pancreatic insufficiency may all be related to inefficient absorption
of vitamin E. In a study by Gallo-Torres et al. (1971), it was found
that the absorption of vitamin E varied with the fat content of the
diet. Serum concentrations of vitamin E were found to be generally
logarithmically related to dietary intake, with ten-fold higher
ingestion leading to doubled serum concentrations of vitamin E. It
was also found that a higher percentage of dietary saturated

triglycerides promoted a correspondingly higher absorption of vitamin
E, whereas an increase in linoleic acid, or other polyunsaturated
fatty acids, depressed the absorption of vitamin E. In their work
it was found that 80 to 90 percent of the vitamin E absorbed into
the lymphatic system was in the form of unesterified tocopherol,
regardless of the dietary lipid in which the vitamin was emulsified,
or the form in which vitamin E was administered, be it either the
acetate or succinate form. This same association between depressed
vitamin E absorption and greater unsaturated fatty acid content of
the diet was reported by Muralidhara and Hollander (1977) who found
that vitamin E is absorbed by a passive diffusion process into the
lymph and is dependent upon micelle formation, which is depressed
in the presence of polyunsaturated fatty acids. Bieri et al. (1977)
have examined the kinetics of uptake of vitamin E and have found
that it exchanges rapidly between plasma and red cells, with an
equilibrium being reached in six to eight hours. There are, however,
several variables which can influence the distribution of toco-
pherol between red cells and plasma. Farrell and Bieri (1975) found
that the plasma concentration of alpha-tocopherol correlates strongly
with the plasma concentration of total lipids, as beta-lipoprotein,
cholesterol, triglycerides, and phospholipids. They showed
that as total lipids in the plasma increase beyond 15 mg/ml,
the concentration of vitamin E in red cells goes down appreciably.
This suggests that individuals suffering from lipoprotein
abnormalities may have reduced red cell vitamin E concentrations
and enhanced red cell hemolysis, as a result of free radical perox-
idation. In order to determine the influence that feeding of in-
creasing amounts of polyunsaturated fatty acids and other saturated
fats had upon serum vitamin E concentration, a study was undertaken
by Bieri et al. (1978) in rats. Alpha-tocopherol was kept at a
constant level between 30 to 50 mg/kg in different dietary
experiments. After eight to ten weeks, tissues were analyzed for
tocopherol and total polyunsaturated fatty acids (PUFA). There was
a tendency for plasma alpha-tocopherol to decrease with increasing
dietary PUFA. Tissue polyunsaturated fatty acid increased as
dietary PUFA increased. The ratio of alpha-tocopherol to PUFA in
several tissues declined more with the first increase in dietary
PUFA than with the second increase. It was concluded that dietary
PUFA has a variable but small effect on lowering the tissue content
of alpha-tocopherol, and that the change in vitamin E status, as
judged by the tissue ratio of alpha tocopherol to PUFA, is primarily
a result of increased tissue deposition of PUFA. This would suggest
then that high polyunsaturated fat diets utilized in the management
of lipoprotein abnormalities, may be resulting in the production of
low tocopherol-to-PUFA concentrations within cellular membranes,
thereby rendering them susceptible to greater lipid peroxidation and
free radical pathology. It appears that vitamin E should be ingested
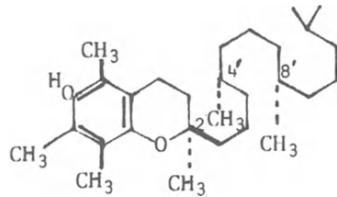in larger amounts when the unsaturated lipid content of the diet is
increased.

Fig. 11. Configuration of d-α-tocopherol (2D, 4'D, 8'D-α-
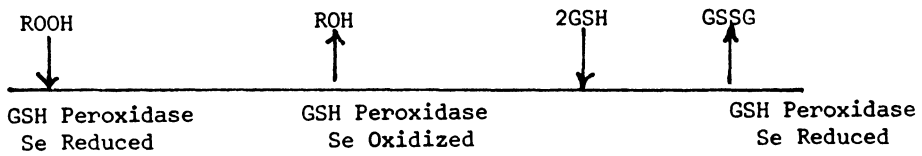   tocopherol).

Figure 12. Reaction of glutathione peroxidase, glutathione (GSH),
            and glutathione disulfide (GSSG).

FORMS OF VITAMIN E AND THEIR BIOLOGIC ACTIVITY

Vitamin E is known to be a vitamin which has several members
in its family, including alpha, beta, gamma, and delta forms of
tocopherol, as shown in Figure 1. Vitamin E is also known to have
three chiral centers, and therefore possesses eight potential
stereoisomers, only one of which is the naturally derived form of
the tocopherols. Synthetic production of alpha-tocopherol will
provide a racemic modification containing 8 stereoisomers. The
α-tocopherol mixture that was formerly considered synthetic, shown
in Figure 11, was called ambo, and can be seen to have only a
configurational change at carbon atom number two. The presently
available synthetic dl-α-tocopherol is totally racemic at all 3
chiral centers and therefore its biological activity has been found
by Ames (1979) to be lower than has been suggested in the National
Formulary. This relationship is demonstrated in Table I. Dam
and Sondergaard (1964) found from a comparison of the natural versus
the synthetic tocopheryl acetates against encephalomalacia that
the potencies were 168 for the d form, 100 for the dl form, and 44
for the unnatural ℓ form of the vitamin. It is important, however,
to recall that the potencies of these compounds as related to their
activities in international units are based upon a specific
biological assay criterion. With regard to vitamin E, the relative
activities have been measured by one of the following methods,
including resorption, gestation in the rat, dialuric acid hemolysis
in the rat, respiratory decline in rat liver, encephalomalacia in
the chick, and exudative diathesis in the chick. As we learn more
about the human activities of vitamin E, it may possibly turn out
that the standard methods of determining biological potency for
vitamin E, utilizing the rat or chick, will be found not to be
suitable assays. Although the alpha form of tocopherol has been
found to be the most potent with 1.38 units per mg of activity in
rat and chick studies, it may be possible that the beta, gamma,
or delta forms of vitamin E play significant roles in human-derived
antioxidant functions. It is, therefore, possible that we have gone
to high therapeutic potencies of the alpha form of vitamin E
commercially at the expense of losing some of the important biolog-
ical activities of the additional forms of vitamin E found in the
natural mixtures, as beta, gamma, and delta.

THE MEASUREMENT OF SERUM VITAMIN E CONCENTRATIONS

In the late 1940s the syndrome of malabsorption and deficiency
of tocopherol were linked (Pappenheimer, 1946; Ansanelli and Lane,
1957). Isolated reports initially associated nontropical sprue
with histologic evidence of avitaminosis E, characterized by ceroid
pigment in smooth muscle of the alimentary tract. Subsequently,
Nitowsky and coworkers (1956a, 1956b) found low blood tocopherol
levels in children with cystic fibrosis, congenital biliary atresia

Table I. Determination of Relative Potencies of Several Forms of α-tocopherol

Form of α-tocopherol	Standard	No. of bioassays	Log relative potency	Relative potency
RRR-α-tocopherol acetate	2-*ambo*-α-tocopheryl acetate	28	0.219	1.66**
RRR-α-tocopherol	2-*ambo*-α-tocopherol	18	0.118	1.31
all-*rac*-α-tocopheryl acetate	2-*ambo*-α-tocopheryl acetate	11	-0.080	0.83*
all-*rac*-α-tocopheryl acetate	*RRR*-α-tocopheryl acetate	19	-0.282	0.52**

* p $\leq$ 0.05 or ** p < 0.01

and, to a lesser extent, cirrhosis of the liver. In a more recent
study Braunstein (1961) found that tocopherol deficiency, as
determined by lowered serum tocopherol concentrations, was
associated with the presence of chronic pancreatitis.

In most early studies the serum tocopherol concentrations were
measured colorimetrically by the Emmerie-Engel method after
elimination of the interference by carotenoids and similar substances
by catalytic hydrogenation (Swick and Bauman, 1952; Quaife and
Biehler, 1945). This procedure, and variants which eliminated
the hydrogenation by a background correction for interfering
carotenoids, was both time-consuming and subject to problems in
maintaining reproducibility. More recently, fluorimetric techniques
have been developed for the determination of serum or plasma toco-
pherol (Taylor et al.1976; Hashim and Schuttringer, 1966). The
method involves saponification of plasma by KOH in the presence of
ascorbic acid. The vitamin E is extracted into spectroscopic-grade
hexane and the fluorescence intensity in the hexane phase measured
at 298nm excitation and 322nm emission at slit widths of 8 and 10nm
respectively. It has been found, however, that contamination of
the sample can occur as a result of exposure to the rubber stopper
of the Vacutainer tube (Sinclair and Slattery, 1978). Whereas
vitamin E values between 1.40 and 1.50 mg/liter were found for all
plasma samples from plastic tubes without Vacutainer rubber stoppers,
those with stoppers were found to be 11.0, 8.7, and 41.5 mg/liter
tocopherol for samples left in contact with red, green and purple
stoppers respectively. Clearly, these results indicate precaution
when blood is collected for sensitive fluorimetric assay of vitamin
E.

The most recent method which may become the method of choice
for serum vitamin E determination is that of high pressure liquid
chromatographic procedures. This technique allows not only the
measurement of total tocopherol concentration, but also the
fractionation of the various forms of tocopherol, be it alpha, beta,
gamma, or delta. The method of DeLeenheer and coworkers (1978,
1979) has allowed for the routine separation of tocopherols in
about 30 minutes utilizing a reverse phase column. This technique
is both fast and free of most interferences and has the additional
advantage of being able to look at the concentrations of each of
the tocopherol positional isomers. It is important to recall that
in the future it may become important to fractionate the tocopherols,
because each has a different biological potency with regard to
specific antioxidant or other physiological effects. Using this
technique, concentrations of the various tocopherols in serum vary
between 6.6 and 15 mg/liter for alpha, 0.0 to 0.2 mg/liter for beta,
and 0.7 to 2.7 mg/liter for gamma. Utilizing high pressure-high
performance liquid chromatography, the determination of serum
vitamin E concentration should be readily obtainable in the clinical

laboratory and provide important information relating to the anti-
oxidant sufficiency of the patient.

LIVER NECROSIS, VITAMIN E AND SELENIUM DEFICIENCIES

     In 1961 Schwarz recognized the Factor Three as a separate
dietary agent, and this product has ultimately been shown to be an
organic selenium compound. It is effective at very small dose
levels and is replaceable in the diet by small amounts of inorganic
selenium compounds. The factor protects against various deficiency
disease, which hitherto have been found to be solely attributable
to a deficiency of vitamin E. Factor Three has been shown to
prevent dietary liver necrosis in the rat and multiple necrotic
degeneration of the heart, liver, muscle, and kidney in the mouse,
as well as fatal exudative diathesis in the chick and in the
turkey (Schwarz and Foltz, 1958). Factor Three, which is derived
in reasonably high concentration from brewers yeast, has ultimately
been suggested to be a mixture of organoselenium compounds which
seem to act as precursors to the active antioxidant enzyme
glutathione peroxidase. This relationship between selenium and the
metalloenzyme glutathione peroxidase represents the first reported
example of the essentiality in humans of the inorganic element
selenium. Selenium, one of the most potent nutritional agents
known, affords protection when added to the diet in the ten-parts-
per-billion range, and the National Research Council suggested
daily dietary intake is from 90 to 120 µg. The element is in the
active site of the enzyme glutathione peroxidase, which is essential
for the maintenance of normal functions and structural integrity of
erythrocytes, liver, kidney, testes, and other organs. It has subse-
quently been found by Schwarz and Pathak (1975) that the organo-
selenium compounds, including the selenodicarboxylic acids, are much
less toxic with higher biologic potencies than the inorganic selenite
or selenate forms. Hoekstra (1975) has found that simultaneous
dietary deficiencies of vitamin E and selenium in weanling rats
result in fatal hepatic necrosis. They found that glutathione
peroxidase activity was reduced by 97 percent in selenium and vitamin
E deficiency in the liver and the oxidation products of lipid
peroxidation, including malonaldehyde, were increased by seven fold,
as compared with rats fed the diet supplemented with selenium and
vitamin E. The decrease in glutathione peroxidase was completely
prevented by selenium administration, but vitamin E had a small
effect. More recently, Lane et al. (1979) have found the effects of
various levels of selenium and vitamin E on glutathione peroxidase
activity in the intestine and liver to be related to whether the
rats were fed a corn/soy bean diet or torula yeast diet. Rats fed
the corn/soy bean diets had greater glutathione peroxidase activity
in the small intestine, colon and liver tissues, catalase activity
and selenium in the liver, and body weight gains were higher than
those fed the selenium deficient-torula yeast diets. Tocopherol
supplementation had no significant effect on glutathione peroxidase

activity in rats fed torula yeast or corn/soy bean diets supple-
mented with selenium, suggesting that selenium does, in fact, play
the selective role in activation of the peroxidase enzyme. Tappel
(1974) has demonstrated that, of the many known interrelationships
between vitamin E and selenium which have been investigated, the
influence of selenium upon the glutathione peroxidase enzyme and
its subsequent effect upon the general chain-breaking antioxidant
properties of vitamin E seems to explain the synergistic relation-
ship between these nutrients. Direct correlation has been found by
Flohe (1971) and Little and O'Brien (1968) between the glutathione
perioxidase enzymes from the tissues studied and their specificity
for the reduction of hydroperoxides through the intermediary of
glutathione as a reductant. The activity of glutathione peroxidase
seems to be directly related to the level of dietary selenium up
to a point of saturation. The influence of glutathione peroxidase
is as shown in Figure 12, where the enzyme is seen to reactivate
glutathione disulfide, a membrane-bound product of free radical
oxidation, to glutathione in order for an additional antioxidation
to occur (Fischer and Whanger 1977). Vitamin E and glutathione
peroxidase work together synergistically to provide a dual mechanism
for protection against lipid peroxidation (Christophersen, 1969;
Schwarz, 1965). The vitamin E, which is lipid soluble and sits
within regions such as the cellular membrane, awaits the production
of an incipient free radical, or hydroperoxide. This hydroperoxide
can be chain radical terminated by abstraction of a hydrogen atom
from tocopherol to produce tocopherol quinone, thereby quenching
the chain radical process. If, however, the vitamin E is ineffec-
tive in dealing with this free radical, the glutatione system can
render the free radical oxidant species into a reduced form which
inactivates it chemically, thereby oxidizing glutathione to gluta-
thione disulfide. In order for the cycle to continue, glutathione
disulfide is then regenerated back to glutathione by glutathione
peroxidase, and the resultant glutathione is now ready to serve as
an additional reducing agent for another free radical. It is,
therefore, clear that vitamin E in conjunction with organoselenium
compounds and the essential amino acid cystine or methionine as a
precursor to glutathione, is essential for the production of an
optimal antioxidant arsenal which protects against in vivo lipid
peroxidation.

SUMMARY

     It can be seen from this discussion vitamin E is a powerful
biological antioxidant, which works in conjunction with other
essential nutritional constituents, such as selenium and the sulfur-
containing amino acids, to affect protection against free radical
peroxidation and pathology in vivo. Considerable evidence has
accumulated in many animal systems that vitamin E deficiency
produces discrete pathology; however, as of yet there is only a

small amount of literature dealing with human chronic vitamin E
insufficiencies and their relationship to histopathologic changes.
The suggested use of vitamin E as a therapeutic agent in certain
human problems, such as respiratory distress in the neonate, and
prevention of retrolental fibroplasia in the premature infant, as
well as the potential use in the autoimmune disease of the adult
human, suggest that both clinicians and fundamental bioscientists
have yet considerable more work to do on both the biological mecha-
nisms and the clinical implications of therapeutic doses of vitamin
E. The data at this time, taken in toto, would suggest that
vitamin E is not an agent which has many of the direct therapeutic
effects in the human that are proven in animals, but that it may
be a significant factor whose deficiency is related to the
progression of many of the lipid peroxidation-induced degenerative
diseases, such as atherosclerosis, connective tissue diseases,
liver necrosis, the myopathies, and potentially even some forms of
cancer, as it relates to carcinogenesis through lipid peroxidation.
At this point in time, these associations are working hypotheses,
however, and not discrete proven causal relationships.

    As a recent review by Chow (1979) has pointed out, cellular
antioxidant mechanisms vary from tissue to tissue. Many metabolic
reactions and dietary components play an important role in cellular
antioxidant defense, many of these may yet remain to be studied.
Further study at the cellular level with humans will be necessary
to have a better understanding of the full clinical importance of
this area.

    The best operative philosophy as it relates to the clinical
application of vitamin E is taken from Leonardo de Vinci, who is
reputed to have generalized, "The supreme misfortune that can
befall any human is for him to embrace a theory, mistaking it for
a fact." The spectrum of activities of the antioxidant nutrients
appears at this point to be quite wide, yet the full unequivocal
demonstration of their effects remains for future work and proof.

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VITAMIN B_{12} AND FOLIC ACID:

CLINICAL AND PATHOPHYSIOLOGICAL CONSIDERATIONS

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INTRODUCTION

The last decade has brought new understanding to the patho-
physiological mechanisms and biochemical interrelationships of
vitamin B_{12} and folic acid and their role in DNA synthesis. The
evolution of facts concerned with these vitamins continues to
unfold in the meticulous fashion begun over 50 years ago (Kass,
1978) when it was recognized that pernicious anemia was a defi-
ciency disease. Even such a momentous contribution at that time
could not foretell the present promise to understand basic cellu-
lar processes.

Both vitamin B_{12} and folic acid deficiency result in an
anemia designated as megalobastic, characterized by blood and bone
marrow changes as a result of DNA synthesis impairment.

The first account of a patient with an intractable anemia is
generally credited to Combe of Edinburgh in 1824 (Chanarin, 1969;
Kass, 1976b). Addison's description concisely presented the clini-
cal findings. Biermer, in 1872, described 15 cases of "progres-
sive pernicious anemia" subsequently analyzed to include six
pregnancy-related anemias, two with steatorrhea, one with chlorosis,
and six with obscure cause, of whom one may have had pernicious
anemia (Kass, 1976b).

In the 100 years since Addison's initial description, the
succession of basic discoveries, adapted from Kass (1976b), in-
clude the following: recognition of related disorders of the
stomach by Flint in 1860; gastric achlorhydria by Fenwick in 1870;
bone marrow changes by Pepper in 1875; peripheral blood cell

characteristics by Quincke in 1876-77, Hayem in 1877, Eichhorst in
1878, and Laache in 1883; increased numbers of erythroid precursors
in the bone marrow by Cohnheim, 1877; designation of megaloblasts
as abnormal erythroid precursors by Ehrlich in 1880; neurological
abnormalities by Leichtenstein in 1884, and by Lichtheim in 1887;
kinetic relationship by Levine and Ladd in 1921; effectiveness of
treatment with liver in dogs by Whipple and Robscheit-Robbins in
1925 and in man by Minot and Murphy (1926); bone marrow changes
with liver therapy by Peabody in 1927; production of a potent
liver extract by Cohn et al. in 1928; determination of an intrinsic
factor in stomach by Castle (1929); isolation of vitamin B_{12}, the
antipernicious anemia principle, by Rickes et al. (1948) and by
Smith in 1948 and purification by Smith and Parker (1948); demon-
stration that extrinsic factor and vitamin B_{12} were the same
(Berk et al., 1948); x-ray diffraction of the molecule and struc-
ture determination by Hodgkin et al. from 1950-56; discovery of
coenzyme B_{12} (5'-deoxyadenosylcobalamin) by Barker et al. (1958);
and synthesis of vitamin B_{12} by Woodward and Eschenmoser in 1972.

Discoveries about folic acid have been no less remarkable.
In the early thirties, Wills (1931, 1932, 1933), while working in
South India, recognized that the "pernicious anemia of pregnancy",
first described by Osler (1919), and "tropical anemia" were essen-
tially the same. They differed from pernicious anemia in not
responding to purified liver extract but did respond to "marmite",
an autolyzed yeast substance. After the purification of pteroyl-
glutamic acid in 1943 (Stokstad, 1943), crystallization from liver
(Pfiffner et al., 1943), and synthesis and structural identifica-
tion (Angier, et al., 1945), it became apparent that the "Wills
factor" and pteroylglutamic acid were the same. Furthermore, the
vitamin M shown by Day et al. (1945) to correct a deficiency
anemia in monkeys, the vitamin B_c which corrected the deficiency
in chickens, and the "norite eluate factor" essential for growth
of Lactobacillus casei (Snell and Peterson, 1940) were the same as
pteroylglutamic acid. Mitchell et al. (1941) termed the Lacto-
bacillus casei factor, "folic acid", concentrating it from four
tons of spinach.

The apparent difference in physiological response to vitamin
B_{12} and to folic acid emerged with the work of Vilter et al. (1950)
by observations that pernicious anemia patients were not able to
maintain their hematological response and the neurological symptoms
exacerbated with folic acid treatment. The basic interrelation-
ships of folic acid and vitamin B_{12}, best explained by the methyl-
tetrahydrofolate trap hypothesis, see Biochemical Considerations,
(Herbert and Zalusky, 1962), are not completely understood. Other
problems relating to drug interrelationships, to neurological and
psychiatric manifestations, to immune mechanisms, and to genetic
findings have recently promoted intensive research. Development of
and use of new techniques to study biochemical and cellular

mechanisms offers promise to understand DNA synthesis and its involvement in neoplastic change of cells.

CHARACTERISTICS OF MEGALOBLASTIC ANEMIAS

General

Classically, the megaloblastic anemias are characterized by pernicious anemia, better termed Addisonian pernicious anemia, as the fatal disease which Addison and others described no longer inexorably results in death. Of insidious onset and progression, the pancytopenia (anemia, leucopenia and thrombocytopenia) associated with macrocytosis results, in probably 95% of cases, from nutritional imbalance, and thus is reversible.

Herbert (1970a) distinguished between vitamin-deficient and those anemias responsive to vitamins. Lack of vitamin B$_{12}$ and folic acid produce nutrient deficient megaloblastic anemias. The importance of their distinction is paramount, for anemias resulting from vitamin B$_{12}$ deficiency respond, for a time, to folic acid but the neurological problems fail to respond. The determination of the etiologic factor or factors is basic to successful outcome. The possible causes for nutrient deficiency may be categorized in one or more of five situations: inadequate ingestion, inadequate absorption, inadequate utilization, increased excretion, and increased requirement (Herbert, 1973). Table I presents such a classification for vitamin B$_{12}$ and folic acid deficiencies. Clinical nutrition has been defined by de Oliviera (1979) as the medical specialty dealing with the physiopathology, diagnosis, and treatment of nutritional diseases and related problems.

Occurrence

Owing to the development and widespread use of electronically automated peripheral blood counting devices, identification of macrocytic erythrocytes (increased mean corpuscular volume) and thus subsequent recognition of megaloblastosis, with or without anemia, has led to a greater awareness of macrocytic red cells associated with various disease processes (McPhedran et al., 1973), or life styles (Helman, 1973).

The prevalence of nutritional anemias as a world wide problem has been examined recently by Baker and DeMaeyer (1979). Collaborative studies were undertaken by the World Health Organization in seven countries with responsible laboratories utilizing standardized techniques and reference assay materials. Women in the third trimester of pregnancy were chosen as the most likely to show a deficiency state as a result of their increased requirements. Generally, the women were from lower socio-economic groups in hospital or clinic settings. Hemoglobin levels were below

Table I. Etiologic Classification of Megaloblastic Anemias in Man
          (modified from Herbert (1979), Rosenberg (1976), and
          Stebbins and Bertino (1976))

Inadequate Ingestion

Vitamin B_{12} Folic Acid

1. Diet lacks sources (micro-   1. Diet lacks adequate
   organisms and animal foods)   sources of unprocessed,
                                     fresh or slightly cooked
                                     foods
   a. Vegans   a. Chronic Alcoholics
   b. Chronic Alcoholics   b. Drug Addicts?
   c. Drug Addicts?   c. Poverty
   d. Poverty   d. Food Faddism
   e. Food Faddism   e. Religious Commitment
   f. Religious commitment   f. Tropical or non-
                                        tropical megaloblastic
                                        anemia

Inadequate Absorption

Vitamin B_{12} Folic Acid

1. Gastric Intrinsic Factor   1. No comparable factor to
   Secretion   vitamin B_{12}?
   absent or inadequate   Presence of folate
                                    binders?
   a. Addisonian Pernicious
      Anemia
      1) Not associated with
         antigen-antibody
         stimulus
         a) Hereditary absence
            of IF
         b) Congenital defect
            of IF molecule
         c) Adult Pernicious
            Anemia

    2) Associated with antigen-
        antibody stimulus
        a) Juvenile Pernicious
            Anemia
        b) Hereditarily deter-
            mined gastric atrophy,
            about one-half of
            adult Pernicious
            Anemia
        c) Acquired gastric
            atrophy
        d) Gastric damage associated
            with endocrine disorders
            and autoimmune diseases
        e) IF Blocking antibody
        f) IF-Vitamin B$_{12}$ complex
            binding antibody
    3) Gastrectomy, total or
        subtotal
    4) Lesions destructive to gastric
        mucosa
        a) Chemical corrosives
        b) Disease processes

2. Ileal Associated Factors
    Resulting in Malabsorption

    a. Enteropathy (less common)

        1) Gluten-induced
        2) Celiac Disease
        3) Idiopathic Steatorrhea
        4) Non-tropical Sprue
        5) Tropical Sprue
        6) Regional Enteritis
        7) Strictures
        8) Surgically induced by
            resection or anastamosis
        9) Malignancy, granuloma,
            or other small bowel
            disease

2. Jejunal or Ileal
    Associated Factors Re-
    sulting in Malabsorption

    a. Enteropathy (more
        common)

        1) Gluten-induced
        2) Celiac Disease
        3) Idiopathic
            Steatorrhea
        4) Non-tropical Sprue
        5) Tropical Sprue
        6) Regional Enteritis
        7) Strictures
        8) Surgically induced
            by resection or
            anastamosis
        9) Malignancy, granu-
            loma, or other
            small bowel disease,
            scleroderma,
            amyloid, diabetic
            enteropathy,
            Whipple's Disease

b. Drugs
1) Para-amino-salicylic acid
2) Colchicine
3) Neomycin
4) Ethanol
5) Metformin
6) Oral contraceptive agents?
7) Calcium-chelating agents

c. ph >6

1) Pancreatic exocrine secretion deficient
2) Zollinger-Ellison Syndrome

d. Disorders of Intestinal Receptors for IF-B$_{12}$ Complex and/or Transport Across the Intestinal Cell
1) Congenital (Imerslund-Gräsbeck Syndrome)
2) Acquired
   a) Absent
   b) Nonfunctioning

b. Drugs
1) Dilantin
2) Primidone
3) Barbiturates
4) Ethanol
5) Metformin
6) Oral contraceptive agents
7) Nitrofurantoin
8) Phenformin
9) Tetracycline
10) Glutethimide
11) Cycloserine

c. Malabsorption with other Disorders
1) Systemic Bacterial infection
2) Dermatitis herpetiformis
3) Cardiac Failure
4) Zinc Deficiency?

d. Disorders of Intestinal Receptors?

e. Congenital and acquired nonconjugase defects

f. Inadequate biliary or intestinal conjugase

g. Conjugase inhibitors in food

3. Competition for vitamin B$_{12}$ by parasite or bacteria

   a. Blind Loop Syndrome
     1) Small bowel diverticuli
     2) Small bowel anastamosis

   b. Fish Tapeworm

4. Radiation Therapy?

3. Competition for Folates by Bacteria

   a. Blind Loop Syndrome
     1) Small bowel diverticuli
     2) Small bowel anastamosis

4. Radiation Therapy?

Inadequate Utilization

Vitamin B$_{12}$

1. Vitamin B$_{12}$ Antagonists

   a. Substituted vitamin B$_{12}$ amides and Anilides
   b. Cobaloximes

2. Congenital or Acquired Enzyme Deficiency or Deletion
   a. Methylmalonyl-CoA Mutase
   b. Methyltetrahydrofolate-homocysteine methyl-transferase
   c. B$_{12a}$ reductase
   d. B$_{12r}$ reductase
   e. Deoxyadenosyl transferase
   f. Other enzyme deficiency or deletion

Folic Acid

1. Folate Antagonists (dihydrofolate inhibitors)
   a. Methotrexate
   b. Pyrimethamine
   c. Triamterene
   d. Pentamidine
   e. Trimethoprim

2. Congenital or Acquired Enzyme Deficiency or Deletion
   a. Formimino transferase
   b. Dihydrofolate reductase
   c. Methyl tetrahydrofolate transmethylase
   d. 5-10-methenyltetrahydrofolate cyclohydrolase
   e. 5-10-methylene tetrahydrofolate reductase
   f. Cystathionine synthetase?
   g. Other enzyme deficiency or deletion

3. Congenital or Acquired Deficiency, Abnormality or Deletion of Transcobalamins (Vitamin B_{12} Binding Proteins)
   a. Abnormal TC I and/or TC III glycoprotein with myeloproliferative disease
   b. Increased TC II with liver disease
   c. Abnormal TC with hepatoma, cirrhosis
   d. Congenital lack of TC II

4. Liver Disease

5. Protein-calorie malnutrition?

6. Malignancy

7. Renal Disease

8. Thiocyanate Intoxication

3. Congenital or Acquired Abnormality of folate binders?

   a. Increased binding by lithium
   b. Increased binding in chronic myelocytic leukemia

4. Liver Disease

5. Vitamin B_{12} Deficiency

6. Ethanol

7. Excess dietary glycine and methionine

8. Diphenylhydantoin and other anticonvulsants

9. Ascorbic acid deficiency

10. Isoniazid

11. Homocystinurias

Increased Requirements

Vitamin B_{12}

1. Physiological
   a. Pregnancy
   b. Infancy

2. Metabolic
   a. Hyperthyroidism?
   b. Chronic Temperature elevation?

3. Increased Hematopoiesis?

Folic Acid

1. Physiological
   a. Pregnancy
   b. Infancy

2. Metabolic
   a. Hyperthyroidism?
   b. Chronic Temperature elevation?

3. Increased Hematopoiesis
   a. Myeloproliferative Disorders
   b. Hemolytic Processes
   c. Chronic Blood Loss

4. Malignancy?

5. Megadoses of Vitamin C?

4. Destruction by Dietary
    Oxidants?

5. Lesch-Nyhan Syndrome

Increased Excretion

1. Lack of, or inadequate TC II

2. Liver Disease

3. Renal Disease?

1. Vitamin B$_{12}$ deficiency?

2. Liver Disease?

3. Kidney Dialysis

4. Chronic exfoliative
    dermatitis and
    psoriasis

Inhibition of or Interferences with DNA Synthesis

1. Interference with purine synthesis or metabolism

    a. Purine Antagonists
        1) 6-mercaptopurine
        2) Thioguanine
        3) Azathioprine

    b. Congenital enzymatic defect in purine synthesis
        1) Lesch-Nyhan Syndome

2. Interference with pyrimidine synthesis

    a. Pyrimidine Antagonists
        1) 5-Fluorouracil
        2) 5-Fluorodeoxyuridine
        3) Hydroxyurea
        4) Cytosine Arabinoside
        5) 6-Azauridine

3. Interference with DNA synthesis?

    a. L-Asparaginase
    b. Azulphidine
    c. Arsenic
    d. Benzene

4. Inhibition of Ribonucleotide Reductase

    a. Cytosine Arabinoside
    b. Hydroxyurea
    c. Iron deficiency
    d. Procarbazine

5. Inhibition of Protein Synthesis

    a. L-Asparaginase

Unknown Mechanisms in Diseases Sometimes

Associated with Megaloblastic Anemia

1. Pyridoxine-responsive anemias

2. Thiamin-responsive anemias

3. Myeloproliferative diseases

    a. Polycythemia vera
    b. DiGuglielmo's anemia

4. Miliary Tuberculosis

5. Aplastic anemia

11 g/dl in from 21.8% (Poland) to 82% (Burma). There was no defi-
nition of the causes of the anemia. Correlations were found
between hemoglobin concentrations and serum iron or the per cent
saturation of transferrin, and with hemoglobin concentrations and
serum folate values. No correlation was found with serum vitamin
B$_{12}$ levels.

The National Nutrition Survey in California (1971) studied a
random sample drawn from census enumeration districts which had a
high per cent of low-income families. Serum folate was below 3
ng/ml (deficient level) in from 5.7% to 20.5% of all ages of sub-
jects. The proportion with unacceptable values (<6 ng/ml) was
greater for serum folate, up to 71.5%, than for any of the other
indices of nutritional status used. By age groups, hematocrit
levels were deficient and low in from 5.6% to 23.5% of all groups.
For all subjects, the serum folate was deficient and low in 64.3%
and the hematocrit in 17.8%.

Clinical Manifestations

The classical accounts of clinical findings in "idiopathic"
anemia continue to hold for some patients in late presentation of
pernicious anemia. Symptoms and findings include: weakness,
lethargy, anorexia without cachexia, dyspepsia, smooth tongue,
glossitis, constipation, peripheral nerve loss with paresthesias,
loss of vibratory sense, forgetfulness, visual loss, mild icterus,
hyperpigmentation which is noticeable even in pigmented races,
extreme pallor, and evidence of cardiac decompensation. All of
which insidiously progress to the point of complete debility and
finally death in untreated patients. Variation of symptomatology
and severity depend on severity of the anemia, its duration, and
cause.

Clinical symptomatology for other causes of megaloblastic
anemia may relate to the underlying mechanism, as in sprue and the
malabsorption syndromes where persistent diarrhea and weight loss
become prominent. Likewise in the increasingly identified, but
rare, hereditable syndromes, the early onset in infancy with fail-
ure to thrive may be a clinical clue. More common are the megalo-
blastic anemias associated with chronic alcohol intake, with preg-
nancy, and with other factors such as chronically administered
medications, e.g., anticonvulsants, contraceptive agents, or anti-
neoplastic drugs. In these situations, the symptoms of the under-
lying process may bring the patient to medical care and interven-
tion before the megaloblastic process has become full-blown.

Morphological Findings

Reviews of cellular morphology in megaloblastic anemias
adequately provide detailed descriptions (Kass, 1976b). The

peculiar characteristics of morphology and staining are attribu-
able to the DNA impairment. All cells are larger than normal;
macro-ovalocytic red cells, macrocytic granulocytes with hyperseg-
mented nuclei, and giant platelets. As the erythrocytic cells
develop, the cytoplasmic portion appears more mature than the
nucleus (nuclear-cytoplasmic dissociation), the nuclear chromatin
retaining a coarse granularity with failure of clumping (Kass,
1976a). Kass (1968) has pointed out the "clock-face" appearance
of the large nucleus in all stages of erythropoietic precursors.
Adherence of separate parts of chromatin to the nuclear membrane
is attributed to result in the dial formation.

Failure to mature also occurs in the white blood cell series.
The cytoplasm appears more basophilic and less mature; the nucleus
becomes pinched off and forms excess segments; the cell is larger
than normal. The thrombocytes also display large size and bizarre
shapes.

Characteristic in the peripheral blood is the macro-ovalocytic
red blood cell, along with hypersegmented neutrophils, low reticu-
locyte count, leucopenia, and thrombocytopenia. In patients with
iron deficiency complicating a megaloblastic process, a dimorphic
situation may appear. Here, there is less evidence of megalo-
blastic change in erythroid precursors, and two populations of red
cells may be seen; the macro-ovalocytes and the microcytic hypo-
chromic cells typical of iron deficiency. After therapy with iron
alone, the megaloblastic changes become evident. Prior to therapy,
the hypersegmented neutrophils or macrocytic metamyelocytes in the
bone marrow may be diagnostically helpful.

Morphological changes occur in other tissues of the body and
may cause diagnostic dilemmas. For example, van Niekirk (1966)
demonstrated that cytological preparations of the uterine cervix
and vaginal tract in women with folic acid deficiency showed
nuclear and nucleoli changes suggestive of carcinoma. Similarly,
mucosal cells of the stomach (Gardner, 1956), and of the intes-
tinal tract (Bianchi et al., 1970; Davidson and Townley, 1977)
show megaloblastic changes which respond to therapy. The smooth-
ness of the tongue has been attributed to megaloblastosis of cells
of the papillary surface (Herbert, 1979).

Ineffective Cellular Production and Increased Cellular Destruction

Finch et al. (1956) have shown that the total erythropoietic
activity in pernicious anemia is about three times normal. However,
the rate of destruction of erythrocytes was equal to or greater than
production. Cellular evidence for intrameduallary destruction was
elucidated by Myhre (1964a, 1964b). Maturation time of megalo-
blasts, a failure of maturation to orthochromic erythroblasts, and
a low incorporation of ^{59}Fe into hemoglobin before therapy

normalized after two or more days following vitamin B$_{12}$ treatment. Other works (Nathan and Gardner, 1962; Astaldi, 1960) have also found maturation time prolonged in untreated pernicious anemia. Failure of DNA synthesis in the G$_2$ phase was postulated as the mechanism leading to erythroid precursor destruction and ineffective erythropoiesis (Wickramasinghe et al., 1967, 1968).

Wickramasinghe and Pratt (1970) have shown ineffective granulopoiesis by autoradiographic and spectroscopic study of granulopoietic cells. A prolonged S period, with up to 30% of giant metamyelocytes showing DNA synthesis retardation, and a premitotic arrest in G$_2$ were found.

Kinetics of platelet metabolism in pernicious anemia, studied by Harker and Finch (1969), demonstrated that despite increased intramedullary megakaryocytic mass in pernicious anemia, the pletelet production and turnover were decreased. Cellular evidence in bone marrow preparations is present in the degenerating megakaryocytes. Harker and Finch (1969) suggested the term, "ineffective thrombopoiesis", be reserved for situations in which daily platelet production per nuclear megakaryocyte unit is less than half that expected. Not only are the platelets quantitatively abnormal but also qualitatively defective. Levine (1973) showed no platelet aggregation response to collagen or to epinephrine prior to therapy in three patients with pernicious anemia. Serial studies on one patient were done. Following therapy with vitamin B$_{12}$, platelet aggregation increased above normal and platelet factor 3 availability, as induced by addition of either ADP or epinephrine, suddenly increased on day nine after therapy. Retrospective study of their 21 cases showed thrombotic episodes in two patients. The latter is not uncommon and is probably a result of increased functional capacity of young platelets and of patient predilection. Ingeborg and Stofferson (1979) confirmed evidence of abnormal platelet function before therapy in pernicious anemia. Although only three of ten patients had a prolonged bleeding time, six had disturbed platelet function. After therapy, one patient developed thrombosis, consistent with the 10% noted by Levine (1973).

That unbalanced growth of megaloblasts is the result of impaired synthesis of DNA, together with premature cell death or abnormal cell division with resultant dysplasia, has been suggested as a "useful model" for the mechanism for megaloblastic transformation by Beck (1977a). Support for this hypothesis was developed: 1) megaloblasts have an even larger cytoplasm than nucleus and hence a larger amount of RNA with relatively unimpaired RNA synthesis; 2) megaloblasts have a normal or slightly increased amount of DNA per cell, accounting for the cytoplasmic basophilia which results from the arrest in the S (synthesis of DNA) phase in order to complete the doubling of DNA before division; and 3) thymidine

and deoxyuridine are incorporated into the DNA of megaloblasts, with thymidine having the capability to correct the megaloblastosis of folic acid deficiency but not of B_{12} deficiency. These factors reinforce the explanation of biochemically defective DNA synthesis and explain the microscopic findings of an imbalance between the young, finely divided, chromatin network and the mature cytoplasm. Lewis and Verwilghen (1973) have presented a review of the various dyserythropoietic anemias and mechanisms of dyserythropoiesis.

Biochemical Considerations

Plasma bilirubin concentrations are moderately increased reflective of the hemolytic component in megaloblastic anemias and result in mild clinical icterus (London and West, 1950). The serum iron and high saturation of transferrin may be the result of the ineffective erythropoiesis; on therapy there is a rapid decrease in serum iron levels with a decrease in the saturation of transferrin. Recent studies indicate that iron may have a definite role in DNA synthesis (Wickramasinghe and Longland, 1974). Absorption of the intrinsic factor-vitamin B_{12} complex by intestinal mucosa involves a bivalent cationic bond, Ca^{++}, with release of vitamin B_{12} in the intestinal cell or on the cell surface upon conversion of the bond (Cooper and Castle, 1960). The sharp decrease of potassium occurring after treatment of megaloblastic anemias may result in cardiac complications and because of this, Lawson et al. (1972) have suggested careful monitoring of potassium during therapy. They found 15 cases of sudden death in a series of 108 patients treated for megaloblastic anemia with hematocrits below 25%. The low potassium could have produced a cardiac arrhythmia, but the causes of death were uncertain.

Zinc has been shown to increase hepatic content of folic acid (Williams et al., 1973) in zinc deficiency. Silink et al. (1975) found zinc an essential part of bovine hepatic conjugase, γ-glutamyl hydroxylase. Tamura et al. (1978) found zinc deficiency led to impaired absorption of dietary folate in six adult men and proposed that intestinal conjugase could be a zinc metalloenzyme. Prasad et al. (1963) found no evidence of folic and deficiency in a population with chronic zinc deficiency. The syndrome of zinc deficiency, including hypogonadism, dwarfism and iron deficiency anemia, was associated with hyperpigmentation and hepatosplenomegaly, two associated findings in pernicious anemia.

Hyperpigmentation is found in many disease states (Greipp, 1978) including endocrinopathies, nutritional deficiencies (pernicious anemia, folic acid deficiency, chronic liver disease), porphyria, autoimmune diseases, after therapy with drugs interfering with sulfhydryl metabolism (busulfan and arsenicals), and with radiation therapy. The biochemical changes resulting in

hyperpigmentation are related to the decreased intracellular glutathionine levels which release melanin synthesis from feedback inhibition of tyrosinase (Gilliam and Cox, 1973). Excess tyrosine levels are found in pernicious anemia (Levine, 1967). As the precursor of melanin, tyrosine is converted by tyrosinase to DOPA which is metabolized to dopa-quinone. A series of non-enzymatic oxidations then produces melanin (Greipp, 1978). Hyperpigmentation in folate deficiency may be explained by elevated biopterin levels as biopterin is involved in the hydroxylation of phenylalanine, necessary in melanin production. Ogbuawa et al. (1978) noted, in three black women with pernicious anemia, that the diffuse palmar, buccal, acral and vulvar hyperpigmentation present at diagnosis regressed with therapy.

VITAMIN B$_{12}$ DEFICIENCY

Occurrence

The highest frequency of pernicious anemia has been recognized for many years among the Northern European peoples and their descendants. Age related, its occurrence increases proportionately (Herbert, 1979). Incidences according to age groups were one per million for ages six months to one year, one per 10,000 for ages one to 10 years, one per 5,000 for ages 30 to 40 years, with progressive increases to one per 200 for the sixth decade of life. Study of population surveys of total cases in the United Kingdom and in Sweden and Denmark showed a range of 100 to 130 cases per 100,000 (Chanarin, 1969).

Kaplan and Rigler (1945) studied autopsy data of 23,231 patients over 45 years of age to determine 293 with pernicious anemia, a frequency of 1.3%.

Chanarin analyzed (1969) the changing age distribution of pernicious anemia in London and Boston over the first half of the twentieth century and found the mean age at diagnosis was 60.5 years where 50 years before, the peak incidence was in the 40 to 50 year age group.

Relatively uncommon in Jews and Orientals, pernicious anemia has also been less frequently seen in blacks than in whites. A greater prevalance than previously thought to exist in blacks in both the United Sates (Hart and McCurdy, 1971; Solanki et al., 1978; Carmel and Johnson, 1978) and in South Africa (Solanki et al., 1978) has been found. It appears to occur at a younger age than in whites (Hart and McCurdy, 1971; Solanki et al., 1978; Carmel and Johnson, 1978) and more frequently in women (Hart and McCurdy, 1971; Carmel and Johnson, 1978; Solanki et al., 1978; Bash and Rosner, 1979). Seventeen per cent of women and 23 per cent of men were under 40 years of age in the Washington, D. C.

group, as compared with 43% for women and 41% for men in the
blacks described from South Africa (Solanki et al., 1978). The
ages of those from Los Angeles and Detroit studied by Carmel and
Johnson (1978), were similar to the Washington, D. C. group. A
higher prevalence for intrinsic factor circulating antibody was
found in both groups: 23 of 24 tested by Carmel and Johnson
(1978) and 85% of 20 black women tested by Solanki et al. (1978).
Metz et al. (1961) reported on three young Bantu women, ages 23,
28, and 34 years with Addisonian pernicious anemia associated with
gastric mucosal atrophy. Genetic or environmental roles may play
a part, but they are unknown at present.

Vitamin B_{12} deficiency associated with specific absorptive
defects, e.g., gastrectomy, small bowel disorders, or others, may
not be common owing to medically recognized risks, preventive
vitamin therapy, and vitamin B_{12} reserves which are adequate for
at least three years in most people. Pernicious anemia has been
observed frequently in association with leukemia (Zarafonetis
et al., 1957; Blackburn et al., 1968; Corcino et al., 1971), with
gastric cancer (Kaplan and Rigler, 1945; Blackburn et al., 1968),
with polycythemia vera or erythremic myelosis (Zarafonetis et al.,
1957; Riddell and Davidson, 1968; Engel and Stickney, 1972), with
myelosclerosis (Hoffbrand et al., 1968), and with a variety of
neoplasms (Larsson, 1962; Lowe, 1976; Fraser, 1969; Burnier
et al., 1976; Arvanitakis et al., 1979).

Dietary deficiencies of vitamin B_{12} may be increasing. As a
result of economic circumstances, food faddism, religious commit-
ment, or other reasons, totally vegetarian diets with no milk,
eggs, meat, or other animal products are being consumed by more
people.

The recognized inborn errors of metabolism of vitamin B_{12} are
quite rare, but the recent advances in diagnosis may reveal more
cases.

Biochemical Considerations

Reviews of the chemical and biological aspects of vitamin B_{12}
may supply references for the major developments in the distin-
guished and remarkable series of studies which have employed the
full range of the basic sciences, clinical medicine and biology
(Castle, 1961; Huennekens, 1966; Stadtman, 1971; Herbert, 1975;
Beck, 1977b; Kass, 1976b).

The structure of vitamin B_{12} bears a porphyrin-like ring, the
corrin portion, linked to a central cobalt atom. This is the only
known instance of cobalt in a vital metabolic system in man.
Lying nearly perpendicular to the planar ring is a 5,6-dimethyl-
benzimidazolyl nucleotide, a unique compound not previously known

in nature. The cyanocobalamin molecule contains a cyano-group which lies above the pyrrole rings which, with one exception, are connected by methylene carbon bridges. Methyl groups or acetamide and propionamide residues almost completely saturate the pyrrole rings.

Corrinoid compounds are numerous. Nomenclature has been systematized (Smith, 1965; IUPAC-IUB, 1966; IUPAC-IUB, 1974).

Two compounds, both alkyl cobalt derivaties, serve as co-enzymes: 5'-deoxyadenosyl cobalamin (Barker et al., 1958; Barker, 1967), and methyl cobalamin (Guest et al., 1962).

Deoxyadenosyl cobalamin mediates the methylmalonyl-CoA mutase reaction to succinyl-CoA and is reduced in vitamin B$_{12}$ deficiency (Gompertz, 1971; Silber and Moldow, 1970).

The block in enzyme reaction leads to an accumulation of metabolic intermediates normally converted to succinyl-CoA and metabolized in the citric acid cycle. Kass (1976b) has found cyto-chemical evidence for increased levels of methylmalonyl-CoA in megaloblasts of untreated pernicious anemia. Other biochemical evidence of methylmalonyl-CoA block are the methylmalonic aciduria, characteristic in untreated pernicious anemia (Cox and White, 1962), and propionic aciduria (Cox et al., 1968). Decreased acti-vity of methylmalonyl-CoA mutase may also be involved in develop-ment of neurological manifestations of vitamin B$_{12}$ deficiency. Frenkel (1973) has demonstrated abnormalities of fatty acid syn-thesis by propionate metabolism in nerve tissue of pernicious anemia patients. Total fatty acid synthesis was decreased from that of normal nerve tissue. In addition it was suggested that the abnormal fatty acids found may be pathogenetic. As early as 1937, (Williams et al., 1937) a relation between abnormal lipid metabo-lism in vitamin B$_{12}$ deficiency and myelin degeneration was postu-lated. In pernicious anemia plasma the neutral fat levels were increased while cholesterol esters and phospholipid were decreased. After therapy these returned to normal.

In the second corrinoid coenzyme, a methyl group is the ligand of cobalt (Beck, 1977B). Methyl cobalamin is the prosthetic group (Loughlin et al., 1964) for the N^5-methyltetrahydrofolate-homo-cysteine methyltransferase enzyme and is responsible for conversion of homocysteine to methionine. N^5-methyltetrahydrofolate is the major methyl donor in the biosynthesis of methionine (Guest et al., 1962; Guest et al., 1964; Larrabee et al., 1961; Larrabee et al., 1963).

The methyltetrahydrofolate trap hypothesis is at present the most accepted theory for the mechanism of the N^5-methyltetrahydro-folate transferase enzyme. In vitamin B$_{12}$ deficiency, there is an

accumulation of methyltetrahydrofolate and a depletion of other
folates necessary for the synthesis of purines and pyrimidines re-
quired in the synthesis of DNA (Herbert and Zalusky, 1962;
Buchanan, 1964; Buchanan et al., 1964; Johns and Bertino, 1965;
Nixon and Bertino, 1970, 1972; Hoffbrand, 1975; Das and Herbert,
1976; Herbert and Das, 1976; Hoffbrand et al., 1977).

Only by cobalamin-dependent methyl transferase reaction can
methyltetrahydrofolate be converted to other folate derivatives.
As a consequence of reduced methyl transferase activity, methyl-
tetrahydrofolate increases in the body with resultant decreases in
tissue levels of tetrahydrofolate, formyltetrahydrofolate, and
methylenetetrahydrofolate, and thereby the rate of reactions
dependent on these folate coenzymes is slowed. As a consequence,
biosynthesis of purines and thymine, and therefore of DNA, is
impaired.

Hoffbrand et al. (1977) expanded and confirmed these inter-
relationships. Folate polyglutamates are the naturally occurring
folate coenzymes required for folate mediated reactions in amino
acid metabolism and purine and pyrimidine synthesis. As the cir-
culating form of plasma folate is 5-methyltetrahydrofolate, a mono-
glutamate, cells must build the active tetrahydrofolate polyglu-
tamate coenzymes from 5-methyltetrahydrofolate.

Sauer and Wilmanns (1977) point out that while cobalamin
dependent methionine synthesis is the primary metabolic fault in
pernicious anemia, mechanisms other than the methyl folate trap
theory do occur and require other explanations. Among these are
direct cobalamin dependency of folate polyglutamate synthesis, of
folate transfer through the cell membrane, and of thymidylate
synthetase enzyme protein synthesis. Clinically, there are other
objections. Children with defective vitamin B_{12} metabolism and
congential methylmalonic aciduria do not have megaloblastic trans-
formation indicative of impaired DNA. The explanation for the
latter may be afforded by the activity of vitamin B_{12} in the
synthesis of thymidylate synthetase.

Haurani (1973) studied an unusual case of megaloblastic
anemia (pernicious anemia) in whom the serum vitamin B_{12} was 2000
to 3000 pg/ml during relapse although the thymidylate synthetase
activity of cultured phytohemagglutinin-stimulated lymphocytes was
low. Additional pernicious anemia patients, as well as folic acid
deficient individuals and normals, were studied. Only in perni-
cious anemia in relapse were the enzyme values low or absent. This
important work demonstrated for the first time that vitamin B_{12}
deficiency results in a decreased synthesis of thymidylate synthe-
tase and an accumulation of unused 5-methyltetrahydrofolate. As
folate acts as a coenzyme for thymidylate synthetase in DNA syn-
thesis, Haurani noted that vitamin B_{12} could not be the cause of

the megaloblastosis.

Chanarin et al. (1974) and Chanarin and Perry (1977) have pointed out difficulties in accepting the methyl folate trap theory as the total explanation for vitamin B$_{12}$-folic acid inter-relationships. Other reactions in which a cobalamin acts as a coenzyme are those mediated by: glutamate mutase, dioldehydrase, ribonucleotide reductase, glycerol dehydrase, ethanolamine deaminase, ribonucleotide triphosphate reductase, and β-lysine isomerase which utilize 5'adenosyl cobalamin; and in synthesis of methane and of acetate which utilize methyl cobalamin (Beck, 1977b; Herbert and Das, 1976). Beck (1977b) points out that the validity of system dependency on a vitamin B$_{12}$ coenzyme is based on the demonstrated requirement in a cell-free system. Only adenosyl- and methyl- cobalamin have been so designated.

Kass (1976b) has observed cytochemical evidence of histone metabolism abnormality in pernicious anemia and folic acid de-ficiency. The histones are thought to bind DNA into compact masses reseı ling a clock face on the interior surface of the nuclear membrane of megaloblasts (Kass, 1968). Additional evidence is based on the preponderance of lysine-rich and lack of arginine-rich histones in pernicious anemia megaloblasts.

Aberrant chromosomal patterns of bone marrow cells in un-treated pernicious anemia demonstrate unusual metaphase configura-tions, chromosomal gaps, and constrictions (Kiossoglou et al., 1965; Powsner and Berman, 1965; Menzies et al., 1966).

Nutritional Requirements of Vitamin B$_{12}$

Vitamin B$_{12}$ is unique in that only foods of animal origin supply the dietary needs. The ultimate source of vitamin B$_{12}$ in animals depends upon microbial synthesis.

The "average" daily diet in the U.S.A. contains between 3 μg and 30 μg vitamin B$_{12}$ depending on the proportion of animal food-stuffs of which between 1 μg and 5 μg are absorbed (WHO, 1972; Heyssel et al., 1966). The average daily requirement for adult men and nonpregnant women has been estimated between 0.1 μg to 1 μg (Herbert, 1968; WHO, 1972). The 0.1 μg/day requirement is based on data in which daily intramuscular injections of 0.1 μg vitamin B$_{12}$ given to pernicious anemia patients resulted in a slow return to normal serum vitamin B$_{12}$ levels following a suboptimal reticulocyte response. Optimal responses occurred with daily in-jections of 1 μg (West and Reisner, 1949; Sullivan and Herbert, 1965).

Studies using whole body turnover of radioactive tagged vitamin B$_{12}$ in normal individuals who were in a steady state

provided a range of 0.5 to 2.5 µg per day (Gräsbeck, 1960; Heinrich, 1964; Heyssel et al., 1966; Reizenstein, et al., 1966). Darby et al. (1958) had shown earlier in a patient with pernicious anemia in relapse the range of utilization of vitamin B_{12} was 0.5 to 2.0 µg/day.

The most recent edition of the NRC recommended daily dietary allowances (1974) has set the daily level of vitamin B_{12} intake "for maintenance of good nutrition of practically all people in the U.S.A." at 3 µg for both sexes over the age of 10 years with an additional 1 µg per day for pregnant or lactating women. Allowances set for infants are based on approximations of vitamin B_{12} in human milk and amount to 0.3 µg daily. Interpolation of values from 0.3 µg at one year of age to 3 µg at 10 years of age have been made.

Young and Scrimshaw (1979) discuss genetic and biologic variables which affect human nutrient requirements. Individual requirements for vitamin B_{12} are affected by these variables. Beck (1977b) considered allowances in relation to obligatory loss approximating 0.1 per cent of the total body pool, as shown by Heyssel et al. (1966), and suggests the minimal daily requirement is adequate to maintain nutritional balance only in subjects with low body stores.

A diet usual in Western countries is believed to be adequate to gradually replenish depleted body stores.

Absorption of Vitamin B_{12}

Absorption of vitamin B_{12} occurs by two mechanisms: simple diffusion, accounting for less than three per cent of the usual range of 1 µg to 5 µg absorbed, and by complexing with intrinsic factor (IF). The latter is the significant absorptive mechanism whereby vitamin B_{12} derivatives, released from food by peptic digestion (Doscherholmen and Swaim, 1973), are attached to specific binding sites of gastric IF (Castle, 1929; Castle and Townsend, 1929; Castle et al., 1930; Gräsbeck, 1959) secreted by parietal cells (Hoedemaker et al., 1964; Taylor, 1965; Jacob and Glass, 1969; Jacob and Glass, 1971). The IF-B_{12} complex is required for absorption by specific absorptive sites on the ileal mucosal cells (Booth and Mollin, 1959). The ileal receptors in man are capable of binding 0.5 µg to 1.0 µg vitamin B_{12} bound to IF (Hooper et al., 1973). Katz and Cooper (1974) have determined the rate-limiting factor in binding of the IF-B_{12} complex is the number of ileal receptors or other intestinal process. A specific site on the IF molecule binds with the ileal receptor in the presence of Ca^{++} (Herbert, 1959) and at neutral pH (Herbert and Castle, 1961; Mackenzie and Donaldson, 1972; Hooper et al., 1973).

Anti-IF antibodies of two types have been identified. The "blocking" type prevents binding of vitamin B$_{12}$ by IF. The "binding" type combines with the IF-B$_{12}$ complex or with the free IF without impairing its ability to bind vitamin B$_{12}$ (Kass, 1976b) and prevents attachment to ileal receptors.

Parietal cell antibodies have also been identified in pernicious anemia. The evidence for autoimmune mechanisms and the state of knowledge has been reviewed by Taylor (1976) and Kass (1976b).

The vitamin, released from the IF complex, passes into the cell or is incorporated into the cell by pinocytosis (Beck, 1977b) and then dissociates in the cell. From the cell it enters the portal circulation and combines with the transport proteins (Allen, 1975) to be delivered to liver, bone marrow and other tissues.

The enterohepatic circulation of vitamin B$_{12}$ is important in the body economy. Reizenstein (1959) found 3 µg to 9 µg vitamin B$_{12}$ per 24 hours were recirculated in the bile. All but approximately 1 µg was reabsorbed. As a result of the biliary reabsorption, 10 to 12 years are required for a person on a strict vegetarian intake to become devoid of vitamin B$_{12}$ (Heyssel et al., 1966) and to evidence clinical deficiency. For a person lacking in IF but with normal B$_{12}$ stores, one to four years after withholding parenteral vitamin B$_{12}$ supplementation would be required. In patients with ileal dysfunction or other cause for malabsorption, vitamin B$_{12}$ deficiency occurs more rapidly as a result of non-reabsorption of biliary vitamin B$_{12}$.

Inadequate secretion of IF may accompany lesions which destroy the gastric mucosa; ingestion of corrosives, linitis plastica, neoplastic lesions; lesions which accompany gastric atrophy; and other conditions such as iron deficiency, multiple sclerosis, subtotal or total gastrectomy, and certain endocrine disorders.

Inadequate absorption of the IF-B$_{12}$ complex may result from any condition interfering with ileal function: tropical sprue, non-tropical sprue (idiopathic steatorrhea, gluten-induced enteropathy), regional enteritis, ileal resection, neoplasias, granulomas, ileal strictures and anastamoses.

Pancreatic insufficiency results in malabsorption of vitamin B$_{12}$, which is correctable by pancreatic extracts and trypsin (Toskes and Deren, 1972; Toskes et al., 1971; Toskes et al., 1973). Low intestinal pH of the small bowel which accompanies pancreatic disease interferes with absorption of vitamin B$_{12}$. Sodium bicarbonate therapy enhances absorption in this situation by raising intestinal pH.

Malabsorption is commonly found in untreated pernicious anemia patients, which is reversible with treatment. In three of four pernicious anemia patients studied by Brody et al. (1966) the malabsorption was associated with sprue, with jejunal diverticuli, or with side to side entero-enterostomy. In the fourth patient no associated abnormality was found. In additional studies on these patients (Carmel and Herbert, 1967), the malabsorption was corrected after B_{12} therapy continuing for one to ten months, suggesting that the malabsorption could be related to ileal damage associated with vitamin B_{12} deficiency. Lindenbaum et al. (1974) studied intestinal function in 28 untreated pernicious anemia patients. D-xylose absorption was impaired in 29%, fat in 9%, and vitamin B_{12} given with intrinsic factor in 75%. Two patients had high titers to serum IF antibodies. The malabsorption (xylose) continued from 2 weeks to 41 months after vitamin repletion. The work supports the hypothesis of Herbert (1972c) that a "vicious cycle" may occur with secondary malabsorption aggravating the pernicious anemia.

Mathan et al. (1974) have found that cobamides with unaltered corrin ring and an intact benzimidazole moiety competitively inhibit the binding of cyanocobalamin to IF. In addition, they reported the nature of the cobamide bound to IF had little effect on the binding of IF to the ileal mucosal receptor.

Mechanisms of action in the vitamin B_{12} absorptive process by IF, so brilliantly elucidated by Castle, and the conditions responsible for the lesions, continue to challenge researchers. While an immune mechanism is strongly suspected (Taylor, 1976) in absorption impairment, additional exogenous as well as endogenous factors may be contributory.

Transport of Vitamin B_{12} - The Binding Proteins

Since Ternberg and Eakin (1949) identified a non-dialyzable substance from gastric juice and hog intrinsic factor which bound vitamin B_{12}, considerable knowledge has developed of the nature and activity of protein-binding of the vitamin. Extensive reviews have brought forward the clincial and scientific importance of this group of proteins (Gräsbeck, 1969; Hall, 1971; Gräsbeck, 1975; Allen, 1975).

All human body fluids that have been studied contain proteins that bind vitamin B_{12} (Gräsbeck, 1969). Only found in gastric juice and stomach extract, IF is a glycoprotein which avidly binds the vitamin. Other vitamin B_{12} binding proteins have undergone brisk investigations primarily as a result of improved conventional techniques (Gräsbeck et al., 1966; Visuri and Gräsbeck, 1973) and development of sophisticated new techniques, such as affinity chromatography (Allen and Majerus, 1972a,b,c; Allen and Mehlman, 1973;

Christensen et al., 1973), isoelectric focusing (Stenman, 1971),
and a method to evaluate structural variants of Transcobalamin (TC)
II (Frater-Schröder et al., 1979).

A new nomenclature of the three types of binding proteins has
been suggested by Gräsbeck (1969, 1975). These are 1) IF, 2) R-
type binding proteins or cobalophilin, and 3) TC II. "R-type"
binding proteins attained the name by "rapid" electrophoretic
mobility for the B$_{12}$ binding protein in gastric juice. Immunologi-
cally identical binding proteins in other tissues, including saliva,
tears, granulocytes and other tissue, were subsequently found but
lacked rapid mobility. Cobalophilin has been suggested to replace
the term "R-type binding proteins".

The three classes of vitamin B$_{12}$ transport proteins have simi-
larities in that they contain single polypeptide chains of 340 –
375 amino acids with each chain containing a single B$_{12}$ binding
site. However, they do not cross react with each other immunolo-
gically (Allen, 1975). They differ in amino acid and carbohydrate
composition, in molecular weight, and in their interactions with
the vitamin B$_{12}$ molecule. That these proteins are coded by differ-
ent structural genes is supported by documented cases with congeni-
tal deficiencies or structural abnormalities (Hall, 1973).

TC II is the transport protein which carries the exogenous
vitamin B$_{12}$ to all tissues and facilitates entry into the cells
(Finkler and Hall, 1967; Allen, 1975; Frater-Schröder et al., 1979).
TC II is found in semen and CSF as well as plasma. TC II is
thought to be necessary in CSF for the delivery of vitamin B$_{12}$ to
cells of the nervous tissue (Friedman et al., 1977). Children with
an inherited defect of TC II may develop normally in utero as the
human placenta has a high affinity binding site for TC II. An
earlier study (Boger et al., 1957) demonstrated higher vitamin B$_{12}$
concentration in cord blood than in maternal blood. However,
children with TC II deficiency subsequently develop signs of B$_{12}$
deficiency within a few months after birth. Familial studies
support the finding of Hakami (1971) in two siblings that the ab-
normality is related to a recessive mode of transmission. A signi-
ficant study of transport of therapeutic vitamin B$_{12}$ in congenital
TC II deficiency has been reported (Hall et al., 1979).

About eight hours after an oral dose of radioactive vitamin
B$_{12}$, radioactivity begins to transfer from TC II to TC I
(Doscherholmen et al., 1959; Cooper and White, 1968). According to
prior nomenclature, so-named TC I and TC III are now considered in
the cobalophilin group in which the endogenous vitamin B$_{12}$ is
carried (TC I) and from which bound vitamin B$_{12}$ is slowly trans-
ferred to tissues. TC III has been shown to be derived from granu-
locytes by Burger et al. (1975).

Abnormalities of all vitamin B_{12} binding proteins have been found. Hall (1973) noted inherited IF deficiency resulting in vitamin B_{12} malabsorption. A biologically inert IF was described in a 13 year old boy whose parents were first cousins (Katz et al., 1972). The parents and sister of the patient were heterozygous for the abnormal IF (Katz et al., 1974).

Cobalophilin (TC I) levels are elevated in chronic myeloproliferative disorders, particulary chronic myelocytic leukemia (Carmel, 1975), in polycythemia vera (Hall and Finkler, 1969), in metastatic cancer (Carmel, 1975), and in adolescent hepatoma (Waxman and Gilbert, 1973). Jacob et al. (1977) have identified in a patient with bronchogenic carcinoma an abnormal macromolecular complex which bound 90% of the endogenous vitamin B_{12}. Distinct from cobalophilin, they suggested the complex was an immunoglobulin of polyclonal origin. Allen (1975) suggested that increased synthesis of R-type proteins, as well as prolongation of plasma survival as a result of decreased liver clearance, may be the mechanisms for elevations in myeloproliferative and neoplastic diseases.

Gräsbeck (1975) predicted additional causes of vitamin B_{12} deficiency may be found as a result of autoantibodies directed against cell membrane receptors and mitochondria.

Clinical Manifestations of Vitamin B_{12} Deficiency

Other than the general findings of a megaloblastic anemia as described in Clinical Manifestations, vitamin B_{12} deficiency has distinctive characteristics. Increasingly, it is diagnosed as a result of newer knowledge indicating that deficiency occurs in persons other than the typical middle or older-aged individual of Scandinavian or Northern European descent. These include persons with inherited abnormalities of absorption, transportation, and with increased vitamin B_{12} requirements as well as persons with environmentally conditioned deficiency. Some of these are: vegans (strict vegetable diet), alcoholics (more often folic acid deficiency occurs), and accompanying other diseases such as auto-immune diseases, hyper- and hypothryoidism, hypergammaglobulinemia (Burnier et al., 1976), hypogammaglobulinemia (van Dommelen, et al., 1963), and endocrine disorders. Vitamin B_{12} is also found to be deficient after gastrectomy (Deller and Witts, 1962; Deller et al., 1962; Hines et al., 1967), in the blind loop syndrome (Dellipiani et al., 1968; Booth et al., 1968; Donaldson, 1968; Brandt et al., 1977), in cystic fibrosis (Deren et al., 1973; Rucker and Harrison, 1973), with pancreatic insufficiency (Von der Lippe, 1977), with massive small bowel diverticulosis (Badenoch et al., 1955), and with bacterial overgrowth in the small bowel (Brandt et al., 1977). The latter has demonstrated that gut bacteria, in particular E. coli, convert vitamin B_{12} to non assimilable analogues altered on

the nucleotide portion of the molecule. The analogues competitive-
ly inhibit the binding of vitamin B$_{12}$ to IF.

Formerly, infestation by the fish tapeworm (Diphyllobothrum
latum) was an important etiology for vitamim B$_{12}$ deficiency in
Scandinavian and Far Eastern countries. However, with gradual
dying out of the fish as a result of water pollution and less raw
fish consumption, this is no longer so frequently seen. Nyberg
(1960) demonstrated the possible inhibition of absorption of IF-B$_{12}$
complex in this clinical situation. More recent studies indicate
the defect is a result of competition for vitamin B$_{12}$ by the worm
(Beck, 1977b).

Malabsorption of vitamin B$_{12}$ is also caused by certain drugs
(Waxman et al., 1970b; Herbert, 1972a). Para-amino-salicylic acid
used in treatment of tuberculosis, colchicine for gout, neomycin
for certain infections, and ethanol are the most important causes
for drug induced malabsorption. In addition, the biguanide group
of oral hypoglycemic agents produce vitamin B$_{12}$ malabsorption but
are no longer available as the FDA restricted their use as a
result of danger from lactic acidosis production. To what extent,
if any, the oral contraceptive agents produce vitamin B$_{12}$ malab-
sorption is presently unknown. Herbert and Jacob (1974) also
cautioned that intake of 500 mg or more of Vitamin C may result in
vitamin B$_{12}$ destruction.

Environmentally induced vitamin B$_{12}$ deficiency may become more
prominent as a result of the increasing use of macrobiotic diets
for reason of religious or other commitment. Economics may also
play a part in persons taking a strictly vegetarian diet. Hines
(1966) reported severe anemia responsive to vitamin B$_{12}$ in a 41
year old vegan (no animal product food eaten) of 7 years duration
following a six year period of a lacto-vegetarian diet (diet in-
cluded milk products). He had also been a blood donor, giving a
pint of blood every three to four months. Therapy with 1 µg
vitamin B$_{12}$ intramuscularly per day produced a reticulocytosis of
14.5% as well as a marked reduction in serum iron and a return to
normal serum lactic dehydrogenase activity.

In another case report, a 6 month old, exclusively breast fed
child of a 26 year old vegan mother, presented in coma, with severe
megaloblastic anemia, and hyperpigmentation (Higginbotham et al.,
1978). Biochemical findings included methylmalonic aciduria, homo-
cystinuria, cystathionuria, glycinuria, methylcitric aciduria,
3-hydroxypropionic aciduria and formic aciduria. The child also
had hepatomegaly, gross abnormalities of the EEG, and monilial
dermatitis. By one month after the start of vitamin B$_{12}$ therapy,
all abnormalities had disappeared. In addition, striking increase
in growth occurred with a gain of 5 cm in length and 3 cm in head
circumference.

Clinical and laboratory findings specific to vitamin B_{12} deficiency are neurologic abnormalities, decreased serum vitamin B_{12} levels, methylmalonic aciduria, reticulocytosis after vitamin B_{12} therapy with no response to physiologic doses of folic acid (100-400 μg/day), and abnormal response in deoxyuridine suppression (Killman, 1964,; Herbert et al., 1973). The latter probably offers the greatest clinical potential of any recent development for differentiation of megaloblastic anemias.

Neurologic abnormalities characteristically develop insidiously and symmetrically. First, paresthesias develop in the distal parts of the extremities, with associated loss of vibratory sense and proprioception. In the untreated patient these progress to spastic ataxia and the manifestations of subacute combined system disease of the dorsal and lateral columns of the spinal cord. Accompanying cerebral manifestations of irritability, somnolence, forgetfulness, EEG changes (Samson et al., 1952), and overt psychosis often revert dramatically with vitamin B_{12} before hematologic response is apparent. Optic atrophy may occur. The neurologic lesion is a degeneration of the myelin sheath. Pathogenesis of the degenerative changes is incompletely known. It has been suggested that one possible reason is that adenosyl cobalamin (coenzyme B_{12}) is required for fat metabolism in propionic acid utilization by conversion of methylmalonic acid to succinate (Herbert, 1979), or possibly vitamin B_{12} may facilitate reduction of the S-S form of coenzyme A to the active S-H form.

Once developed, the subacute combined system symptoms usually respond incompletely to therapy. The ability of large amounts of folic acid to mask the blood and bone marrow megaloblastosis, with a suboptimal but significant reticulocytosis, and at the same time to exacerbate the neurologic deficits (Vilter et al., 1950), is incompletely explained but is critical to the proper care of the patient (Carmel, 1979). Because of this danger FDA regulations prohibit the inclusion of more than physiologic levels of folic acid in generally available multivitamin preparations.

Inhibition of cell growth by anesthetic gases, considered in the past to be a narcotic effect, has been shown to interfere with DNA production by depressing incorporation of thymidine and to cause a delay in entry of cells into the DNA-synthetic phase (Sturrock and Nunn, 1974; Nunn et al., 1976). Sturrock and Nunn (1975) have also demonstrated multiple mitoses and multinucleate cells in mammalian cells exposed to nitrous oxide and halothane. Trudell et al. (1979) suggested that nitrous oxide is carcinogenic by its reduction action of aromatic nitro compounds, as determined on mammalian tissue and gastrointestinal microflora both in vitro and in vivo.

Pregnant rats exposed to 45 to 50% nitrous oxide for 2, 4, or 6 days on day 8 underwent resorption of fetal products in 19%, 25%, and 57%, respectively (Fink et al., 1967). Lane et al. (1979) have confirmed the teratogenic nature of nitrous oxide.

Interference with cell division by various anesthetics was demonstrated as long ago as 1914 (Lillie, 1914).

For a limited time in the 1950's, nitrous oxide anesthesia was used to control neurologic symptoms in tetanus infections, but instances of bone marrow failure, with severe leucopenia, thrombocytopenia and bone marrow megaloblastosis, resulted in abandonment of that treatment (Lassen et al., 1956; Sando and Lawrence, 1958). Because of the bone marrow and hematologic effects, it was also studied as a treatment of acute leukemia (Eastwood et al., 1963) and subsequently abandoned as the leucopenic effect was not sustained. In two patients, both of whom died, the polymorphonuclear cell count fell faster than the lymphocytes, and the bone marrow became hypoplastic.

A recent report (Layzer, 1978) documented severe myeloneuropathy in six patients (five dentists and one hospital technician) who had prolonged nitrous oxide exposure. The myeloneuropathy was similar to that seen in subacute combined system disease with numbness and paresthesias in extremities and depressed reflexes. There appears to be a high rate of abuse of nitrous oxide, 20% of medical and dental students reported by Rosenberg et al., (1979). Another report cited three deaths from halothane abuse in hospital personnel (Spencer et al., 1976).

Amess et al. (1978) found eight patients receiving 50% nitrous oxide and 50% oxygen for 24 hours during and following cardiac bypass surgery who developed megalobastic bone marrow findings. Deoxyuridine suppression tests were abnormal at the end of ventilation with the anesthetic gas. Five patients undergoing cardiac bypass surgery, but without nitrous oxide, had normoblastic marrows and normal deoxyuridine suppression tests. Of nine additional patients who received nitrous oxide only during surgery, three had abnormal deoxyuridine suppression tests at 24 hours. The deoxyuridine abnormality was identical in that found in vitamin B$_{12}$ deficiency, but the serum vitamin B$_{12}$ levels were normal. The effect appeared after less than six hours exposure to nitrous oxide in one patient. Recovery occurred on stopping the nitrous oxide. Treatment with hydroxocobalamine before and after exposure did not prevent the abnormality.

In another study, Deacon et al. (1978) exposed rats to 50% nitrous oxide: 50% oxygen and measured liver methionine synthetase, excretion of methylmalonic acid and deoxyuridine suppression.

Methionine synthetase, which requires methyl cobalamin as a co-
enzyme in the conversion of homocysteine to methionine, was
virtually absent in the livers after six hours exposure. Methyl-
malonic acid excretion was not affected by 24 hour exposure. The
deoxyuridine suppression test became abnormal shortly after in-
activation of the methionine synthetase.

An editorial (1978) has evaluated these findings with nitrous
oxide and those with other anesthetic gases. They made the inter-
esting speculation that chemotherapy given during anesthesia may
preferentially affect tumor and spare the host since it has been
reported that transplanted tumor cells were less inhibited in
their proliferation than were normal marrow cells.

A recent abstract (Thom, 1979) indicates, from work with
microbiological systems subjected to anesthetic gases and helium,
that helium, by antagonizing narcosis, enhanced growth of organ-
isms except in the presence of nitrous oxide. In this case,
growth was inhibited.

While the mechanism of action of nitrous oxide in producing
bone marrow failure, DNA synthesis disruption, and neuropathy may
relate to impaired vitamin B_{12} metabolism, continued work is needed
to establish these mechanisms.

The complex nature of the many unsolved questions concerning
neurological findings in folate and vitamin B_{12} deficiency have
been reviewed (Reynolds, 1976). Reynolds postulated that the
hematological and neurological expressions of vitamin B_{12} de-
ficiency have a similar biochemical basis, and that a block in
folate metabolism is responsible for the neurological consequences.

Diagnosis of vitamin B_{12} deficiency rests on the demonstration
of the typical clinical features of megaloblastic anemia, achlor-
hydria after histamine stimulation, and response to physiologic
doses of parenteral vitamin B_{12} (Minot and Castle, 1935; Sullivan
and Herbert, 1965; Sullivan, 1970b; Beck, 1977b; Hall, 1973;
Toskes and Deren, 1973; Glass, 1974; Herbert, 1979). The latter
may be an incomplete response in the presence of active infection,
renal disease, or tumor.

Achlorhydria is present if the pH of the gastric juice is
never less than 3.5 and decreases less than one pH unit after his-
tamine stimulation (Beck, 1977b). Hypochlorhydria is present
should the pH unit decrease by more than one, even though the
initial pH is above 3.5. While Addisonian pernicious anemia is
precluded in the absence of achlorhydria, certain vitamin B_{12}
responsive conditions, especially congenitally acquired absence of
IF, or other rare conditions of absorptive failure may occur in
the presence of free gastric acid.

Additional diagnostic studies are necessary for the more commonly encountered patients presenting with atypical or less severe findings than in late Addisonian pernicious anemia. Indications for additional tests are the uncertainty of the cause of megaloblastic anemia and/or presence of neurologic findings in the absence of anemia. Special tests include measurement of the serum vitamin B$_{12}$, absorption of vitamin B$_{12}$, assay of IF, measurement of abnormal metabolite products, and the deoxyuridine suppression test (Killmann, 1964).

Serum vitamin B$_{12}$ measurements employing microbiological assay by Euglena gracilis or Lactobacillus leichmanii are generally less than 100 pg/ml (normal, 315 ± 125 pg/ml) (Kass, 1976b) Radiodilution assay methods have been developed but appear less reliable than the microbiological techniques (Whitehead and Cooper, 1977; Donaldson, 1978; Cooper and Whitehead, 1978; Kolhouse et al., 1978), because cobalamin analogues normally present in serum may act as inhibitors of cobalamin or may be inert biologically. Kolhouse et al. (1978) suggest this variability may be a clue to further investigation to determine why some patients with vitamin B$_{12}$ deficiency may develop either hematologic or neurologic abnormality whereas in other patients both occur. Important clinical implications arise in the use of radiodilution assay methods. It has been estimated about 20% of the true cobalamin deficiencies are missed with this type of assay since it is extensively used in clinical settings in the form of specialized commercial kits. Kolhouse et al. (1978) feel the error rate may be overestimated as the trained clinician or hematologist should and would obtain additional tests should the clinical and other laboratory findings be in discrepancy.

The Schilling test (Schilling, 1953) evaluates absorption of an oral dose of radioactive vitamin B$_{12}$ by measurement of urinary excretion of the radiolabeled vitamin. In the absence of intrinsic factor or other cause for malabsorption, excretion is less than 5% in the first 24 hours after a "flushing" parenteral dose of non-radioactive vitamin B$_{12}$. If the test is positive, it is repeated utilizing an oral dose of IF with the radioactive vitamin B$_{12}$. A patient with pernicious anemia usually will then excrete more than 5% of the test dose of labelled vitamin. Certain precautions in usage and interpretation are needed. Reliable urine collection is required. The test may be invalid in the presence of vomiting, diarrhea, or renal disease. The large parenteral flushing dose precludes observation of response to physiologic vitamin B$_{12}$ doses. Impaired absorption as seen in sprue, other gastrointestinal disorders (Mahmud et al., 1971; Klipstein, 1972), or other conditions may result in abnormally low excretion both before and after IF.

Herbert (1972b) discussed modifications of the Schilling test technique to elucidate possible malabsorption by measuring amounts

of radioactivity in blood, urine, and feces following 1) oral
vitamin B_{12} test dose, 2) oral dose plus IF, and, if these are
both abnormal, 3) repeating the test after an appropriate trial of
therapy, such as an anthelminthic or antibiotic in the case of
fish tapeworm infestation or blind loop syndrome, respectively, or
after treatment with sodium bicarbonate if pancreatic insufficiency
is present. Herbert emphasized the importance of retesting for
absorption following appropriate therapy to distinguish between
primary or secondary malabsorption.

IF measurements were originally bioassay techniques based on
reticulocytosis in pernicious anemia patients (Minot and Castle,
1935). These prolonged and tedious methods depended on pernicious
anemia patient resources. An in vitro technique developed by
Sullivan et al. (1963) utilized guinea pig ileal homogenate as a
source of receptors for IF. A rapid, less complicated technique
utilizing protein-coated charcoal separation was developed by
Gottlieb et al. (1965). Subsequently, Begley and Trachtenberg
(1979) reported an in vitro assay for IF which eliminated the need
for elaborate forms of protein separation and for a source of
antibody. IF is decreased in partial gastrectomy (Ardeman and
Chanarin, 1966) and may be variable in pernicious anemia (Castle
et al., 1931).

Additional laboratory findings which may be found in associa-
tion with untreated vitamin B_{12} deficiency are elevated serum
folate in 20-35% of patients, elevated serum lactic dehydrogenase,
formiminoglutamic aciduria, methylmalonic aciduria, hyperbiliru-
binemia, increased fecal stercobilin, elevated plasma iron with
increased saturation of transferrin, accelerated plasma iron
clearance, and impaired utilization of iron by red blood cells.
Of the above biochemical abnormalities, the most specific for
vitamin B_{12} deficiency is the excretion of methylmalonic acid;
normal excretion is less than 10 mg/day (Gutteridge and Wright,
1970; Frenkel and Kitchens, 1977). In about 34% of patients with
pernicious anemia, blocking antibodies may be present; about 19%
have both blocking and binding antibodies for IF (Sullivan, 1970a).

Finally, the most recent and probably the most important for
differential diagnosis is the deoxyuridine suppression test intro-
duced by Killmann in 1964. It is particularly useful in recognizing
vitamin B_{12} or folic acid deficiency, specifically differentiating
the two, in non-anemic patients with peripheral blood macrocytosis
and equivocal bone marrow morphology together with normal serum
vitamin B_{12} or red cell folate values (Herbert et al., 1973;
Wickramasinghe and Longland, 1974). The test is based on the fact
that preincubation of normal bone marrow with an appropriate con-
centration of deoxyuridine severely suppresses the subsequent incor-
poration of tritiated thymidine into DNA. The suppression is sub-
normal with vitamin B_{12} or with folate deficiency and is correctable

in vitro by the addition of the appropriate deficient vitamin.

In patients with megaloblastic changes as a result of neo-
plastic, chemotherapeutic, or other agents which interfere with DNA
synthesis, the deoxyuridine suppression test is abnormal. Except
for these, an abnormal test indicates vitamin B$_{12}$ or folic acid
deficiency.

Immune Mechanisms in Vitamin B$_{12}$ Deficiency

Although studied for more than twenty years, basic
mechanisms to account for immunologic aberrations associated with
pernicious anemia continue to elude researchers.

Starting with the approach of Taylor and Morton (1958) in IF
immunized rabbits, Schwartz (1958) then demonstrated IF-inhibiting
substances in sera of pernicious anemia patients treated with oral
hog IF-B$_{12}$ preparations. It had been shown earlier that immuno-
suppressive agents, ACTH and corticosteroids, could increase reti-
culocyte counts or change the bone marrow to produce mature red
cells (Thorn et al., 1950; Wintrobe et al., 1951; Doig et al.,
1957). Ardeman and Chanarin (1965) showed that steroids improved
the vitamin B$_{12}$ absorption in pernicious anemia patients, irrespec-
tive of the presence of antibody to IF. In some, there was a
return of free gastric juice hydrochloric acid and of IF. No
improvement in absorption occurred in gastric resection patients.
An additional pernicious anemia patient with recovery of gastric
mucosal secretion of IF and of acid was reported by Jeffries (1965).

Antibodies to IF and to parietal cells have been demonstrated
in over 50% of pernicious anemia patients (Irvine, 1963; Irvine,
1965). The association of pernicious anemia with autoimmunity was
reviewed by Chanarin (1972). Where the prevalence of pernicious
anemia in Great Britain is one to two per 1000 population, perni-
cious anemia in association with other diseases is 31 per 1000 for
Grave's disease and 108 per 1000 for primary myxedema. Six of 118
with Addison's disease, and seven of 74 with idiopathic hypopara-
thyroidism also had pernicious anemia. Ardeman et al. (1966)
suggested that patients with thyroid disorders should be screened
for pernicious anemia. Other than the possible immune mechanisms
associated with thyroid disease, increased requirements for vitamin
B$_{12}$ have been reported in uncontrolled thyrotoxicosis (Alperin
et al., 1970).

Schade et al. (1966, 1967) demonstrated IF antibodies to be of
two types: precipitating antibody; antibody which combines with IF
before or after addition of vitamin B$_{12}$ and which inhibits vitamin
B$_{12}$ absorption in vivo and in vitro, and blocking antibody; anti-
body which blocks the ability of IF to complex with the vitamin to
form the IF-B$_{12}$ complex. Also termed Type I, or "binding" antibody,

the precipitating antibodies interfere with ileal mucosal absorp-
tion (Glass, 1974), and Type II, or "blocking" antibodies, inter-
fere with coupling of vitamin B_{12} to IF (Garrido-Pinson et al.,
1966; Schade et al., 1966; Schade et al., 1967).

   IF antibodies have also been demonstrated in the absence of
pernicious anemia. In 11 of 23 patients with both thyroid disease
and IF serum antibodies who were followed from three to seven years,
none developed pernicious anemia (Rose et al., 1970). Both types
of IF antibodies have been found in saliva, serum, and gastric
juice. It has been suggested that IF antibodies produced locally
by gastric mucosal cells are of greater importance than serum
antibodies.

   In separate studies, infants found to have no gastric IF at
birth, as a result of maternal transfer. of antibody in utero, sub-
sequently developed IF as the antibody disappeared (Bar-Shany and
Herbert, 1967; Goldberg et al., 1967). Charache et al. (1968) re-
ported an additional infant with normal IF at birth although sub-
jected to maternal anti-IF antibody in utero.

   Parietal cell antibody was reported in 84% of pernicious
anemia patients and in 36% of their healthy relatives (Jeffries
and Sleisinger, 1965; Fisher and Taylor, 1965).

   The heterogeneous nature of the IgG light chain auto-anti-
bodies associated with pernicious anemia, similar tc that of normal
gamma globulin, was described by Bernier and Hines (1967).

   An extensive study of 24 HLA specificities on the A and B loci
by platelet-complement fixation and microlymphocytotoxic techniques
was done on 66 Addisonian pernicious anemia patients by Horton and
Oliver (1976). No association was found for HLA A3, HLA B7, or the
haplotype A3, B7 with the presence of serum antibodies to vitamin
B_{12}-binding sites of IF. A small increase in frequency of the
antigen HLA B8 (38%) was found for those patients who also had
serum thyroid antibodies over that found in thyroid-antibody-nega-
tive patients (13%). The Horton and Oliver (1976) study was unable
to confirm earlier work that HLA B7 was associated with pernicious
anemia (Zittoun et al., 1975).

   Antibodies against TCs have been found in pernicious anemia
patients with high serum levels of vitamin B_{12}. Carmel and Schurafa
(1977) found binding (Type I) antibodies in such a patient.

   Marcoullis et al. (1979) recently reported the first instance
of the simultaneous occurrence of blocking and binding antibodies
to IF, TC II, and TC I and other R-type vitamin B_{12}-binding proteins
in a patient with treated pernicious anemia. Following demonstra-
tion that the dialyzed IgG, but not the IgM, neutralized the

total unsaturated vitamin B$_{12}$-binding capacity of human gastric
juice, saliva, and serum, they suggested that the IgG contained
blocking antibodies (Type II) against IF, TC II, TC I, and other
R-type binders. Binding antibodies (Type I) were also demonstrated.
Where only immuno-complexes of Type II, which prevent vitamin B$_{12}$
from complexing with IF, occur, a high serum vitamin B$_{12}$ is ex-
pected. In this case, the presence of both types I and II anti-
bodies resulted in lower serum vitamin B$_{12}$ levels.

Carmel and Johnson (1978) suggest the earlier age at onset of
pernicious anemia in black and possibly in Latin-American women
may have an autoimmune basis.

FOLIC ACID DEFICIENCY

Occurrence

Folic acid deficiency, next to iron deficiency, is probably
the most common cause of anemia in man. As noted in the etiologic
classification, Table I, causes of folic acid deficiency include
dietary lack of folate-containing foods, as a complication of
gastrointestinal disease, in association with pregnancy, or as a
side effect of certain drugs.

Sauberlich (1977) concluded that the incidence throughout the
world was uncertain, but estimates have probably been understated.
Herbert (1970b) found that as many as 50% of pregnant women have
megaloblastosis by the third trimester, largely in the low-income
population groups who consume basic diets of beans and rice.
Rothman (1970) reviewed 27 studies on pregnant women and noted the
incidence of megaloblastic anemia ranged from 0.5 to 54%. Chanarin
et al. (1965) found a higher incidence of folate depletion in
pregnant women who had not received iron supplementation than in
those who had been supplemented. It must be remembered that it was
in pregnant women in Southern India, that Wills (1931, 1932, 1933)
described the differences in response of megaloblastic anemia to
purified liver extract and to autolyzed yeast which led to the
discovery of folic acid.

In Southern India, Baker and Mathan (1971) found 64% of
patients with tropical sprue had megaloblastic anemia, more than
half of whom had pure folate deficiency. Sprue is known to be
endemic in tropical countries (Klipstein et al., 1966). Herbert
(1968) attributed much of the folate deficiency in Carribean popu-
lation groups to the common practice of cooking foods in boiling
water for a time in excess of one-half hour.

In 95 children with kwashiorkor, studied by Pereira and Baker
(1966), serum folic acid levels were significantly lower (p=0.001)
than in normal children and ranged from 1.4 - 2.4 ng/ml. High

serum vitamin B_{12} levels were also found. Baker and Mathan (1971)
have associated high serum vitamin B_{12} levels in sprue with liver
damage.

     In a study of randomly selected municipal hospital adult
patients, Leevy et al. (1965) found 45% had subnormal folate levels.
Nineteen, of the 117 patients studied, chronically consumed alcohol
to excess; 80 drank socially. Herbert et al. (1963) found up to
80% of alcoholic patients had folic acid deficiency. Further ref-
erences may be found in a review of folic acid deficiency (Streiff,
1970a).

Biochemical Considerations

     Several reviews of the biochemistry of folic acid are available
(Chanarin, 1969; Beck, 1977c; Rosenberg, 1976; Hoffbrand, 1975;
Blakley, 1977).

     Pteroylglutamic acid (folic acid) is the generic substance of
a large group of compounds known collectively as folates. The
molecule (peteroylmonoglutamic acid) is composed of a 2,4,6 substi-
tuted pterin ring, paraminobenzoic acid, and an L-glutamic acid
residue. In nature, folic acid occurs as conjugates in which vary-
ing numbers of glutamic acid residues are attached by peptide
linkages to the γ-carboxyl group of the adjacent glutamic acid
moiety. In liver, the major folate is pentaglutamyl conjugate
(Shin et al., 1972); in plants, heptaglutamyl conjugate (Rosenberg
and Godwin, 1971).

     Folic acid is not active as the monoglutamate in mammals, but
must be reduced by conjugase to release tetrahydrofolic acid, which
serves as the coenzyme in one-carbon transfer acceptance. The
reactions in production of tetrahydrofolic acid are catalyzed by
dihydrofolate reductase, an NADPH-linked enzyme.

     Reduced folic acid derivatives (coenzymes) differ in the one-
carbon unit and its site of attachment. With one exception,
N^5-formyltetrahydrofolic acid, these are unstable to oxidation and
to heat. Alternative names for N^5-formyltetrahydrofolate are
citrovorum factor, leucovorin, and folinic acid.

     Folic acid coenzymes are required in metabolic reactions; pro-
bably the most important of which is the methylation of deoxy-
uridylate to thymidylate in pyrimidine nucleotide biosynthesis,
essential to formation of DNA. If this process is impaired, mega-
loblastosis results. Other reactions for which folate coenzymes
are necessary are the interconversion of serine and glycine (also
requires vitamin B_6), the conversion of histidine to glutamic acid,
the conversion of homocysteine to methionine (also requires vitamin
B_{12}), the formation of glycinamide ribonucleotide, the reaction in

which 4-imidazole carboxamide ribonucleotide reacts with the co-
enzyme to form 5-formamido-4-imidazole carboxamide ribonucleotide,
reduction of dihydrofolate, reduction of 5, 10-methylene tetrahydro-
folate, formiminoglutamic acid conversion to glutamic acid, gener-
ation of and utilization of the formate pool, methylation of uracil
residues in transfer RNA, and folate-dependent methylation not in-
volving S-adenosyl methionine.

Evidence has been found for feedback inhibition and end-pro-
duct repression of folate coenzymes (Blakley, 1977). Krumdieck
et al. (1977) consider the peptide chain of naturally occurring
folates important in the regulation of one-carbon metabolism.
There is evidence that these folates affect passage of coenzymes
across biological membranes, the recognition of proper coenzyme-
carbon-fragment complex, and the activation or inhibition of
enzymes involved in one-carbon metabolism.

Dietary Considerations

Food folates are primarily found in green and leafy vegetables,
fruits, organ meats, and yeast (Toepfer et al., 1951; Chanarin,
1969). The analyses of folic acid in foods are dependent upon
extraction procedures which do not destroy the labile reduced
forms; the assay method used, since microorganisms vary in require-
ments and utilization of one-carbon substituents of tetrahydrofo-
late and the short polyglutamates; and pretreatment with conjugase,
as none of the microorganisms are able to utilize polyglutamates.
Cooking procedures need also to be considered, as excessive boiling
and use of large quantities of water result in folate destruction
(Herbert, 1963).

Need for additional studies on food folate content is recog-
nized as analyses done prior to the use of ascorbate or other sub-
stance to prevent heat lability (Toepfer et al., 1951) may be low.
Evidence also has been found that folates may be non-specifically
bound to cellular fibers (Santini et al., 1962), since, after
removal from fibrous tissue, conjugase treatment of diets results
in an increase of folate assayed. This has not been evaluated for
availability of folate for man.

Tamura and Stokstad (1973) and Stokstad et al. (1977) reported
studies on availability of food folate. Low availability was found
in orange juice, 31%, romaine lettuce, 25%, and egg yolk, 39%.
Higher availability was found in bananas, 82%, dried lima beans,
70%, and frozen lima beans, 96%. High acidity was attributed to
be the cause of low folate availability from orange juice. In
vitro studies by Herbert and Jacob (1974) indicated that pharmaco-
logic amounts of ascorbic acid added to diets assayed for vitamin
B$_{12}$ destroyed 43% of that vitamin.

Rosenberg (1975) studied absorption by man of tritiated mono- and heptaglutamic derivatives of folate. After a flushing dose of non-tritiated folic acid, 70% of the monoglutamate and 56% of the polyglutamate were recovered in the urine in 48 hours. He concluded that dietary folate, 80% of which is in polyglutamate form, was substantially utilized although various foods may differ in efficiency.

Waxman et al. (1970a) evaluated the evidence that amino acids could effect megaloblastosis. Methionine, homocysteine, serine, and glycine are required in the production of DNA thymine. In in vitro human bone marrow culture studies, methionine and glycine provoked megaloblastosis; whereas, homocysteine and serine appeared to reduce the DNA defect.

Hoppner et al. (1977) called for evaluation and standardization of methodology in dietary folate analysis, and additional data for folate content of foods. They concluded from data presently available that from the "average composite" diet, adolescents, pregnant, and nonlactating adults may not obtain adequate amounts of folic acid.

Dietary Folic Acid Requirements

Herbert (1962b) showed that 50 µg folic acid per day was the minimal daily amount needed to maintain serum folate levels in man. The classic depletion study produced megaloblastic changes in 133 days in a normal male on a diet containing 5 µg folic acid per day (Herbert, 1962a) indicating body stores are depleted in about four to five months.

Official NRC-NAS (1974) requirements are set at 400 µg per day to meet needs for good nutrition of adults and double that amount in pregnancy. Infant requirements are 40 to 50 µg per day. Waslein (1977) concluded the FAO-WHO recommendation of 5.0 µg/kg body weight is adequate for the normal child, but for maximal repletion in protein-calorie malnutrition two to three times that amount are needed.

Considerable attention has been given to the study of folic acid requirement in pregnancy. Up to 50% or more demonstrate megaloblastic changes by the third trimester (Herbert, 1970b; Beck, 1977c). In one study, seventy per cent of the pregnant women with folic acid deficiency were multiparas (Stone et al., 1967). The subject has been reviewed extensively (Whiteside et al., 1968; Chanarin, 1969; Davis et al., 1969; Kitay, 1969; Rothman, 1970).

Contributing to the high frequency of folic acid deficiency in pregnancy are physiologically increased requirements, adolescent pregnancies, poor diet, anorexia, infections, coexisting hemolytic

conditions, and drugs such as prior use of oral contraceptive
agents or anticonvulsants.

Complications of hemorrhage, abortion and congential malforma-
tions were noted by Hibbard and Smithells (1965). Stone (1968)
found 60% with abruptio placenta had positive formiminoglutamic
acid excretion. Pritchard et al. (1969) were unable to confirm
these findings. The latter compared the baby's hemoglobin at
birth with that of the mother. For mothers with either more or
less than 6.9 g/dl, the infants' hemoglobins were 18.9 g/dl. In a
patient seen by Beck (1977c) the mother had a megaloblastic anemia
at term with only 6 g/dl hemoglobin level, while the baby, de-
livered before folic acid could be given, had a hemoglobin level
of 28. He attributed the high hemoglobin level to anoxia. Another
explanation may be for folic acid to go preferentially to the
fetus. No firm explanation is available. However, Cooper et al.
(1970) observed an abrupt rise in serum folic acid seven days post-
partum and postulated a shunt across the placenta and also a low
rate of transfer of liver folate to the plasma of the mother.

Routine folic acid supplementation in pregnancy is generally
accepted with amounts recommended varying from 0.5 mg to 1.0 mg
per day.

Another possible complication observed in megaloblastic anemia
in pregnancy is the histological change of the uterine cervix
smears (Kitay and Marshall, 1968; Kitay, 1969) which could possibly
be mistaken for malignant change (O'Brien, 1962; van Niekirk, 1966).
The changes may be a result of localization of conjugase to target
tissues or to other systems whenever there is a cycle of cell mul-
tiplication. Krumdieck et al. (1977) have found the conjugase
activity of the rat uterus is increased three fold at the time of
maximum estrogen secretion. That the uterus is a target tissue
for folic acid is probably borne out in the use of folic acid
antagonists to induce abortions (Thiersch, 1952) which was renounced
by Goetsch (1962) as hazardous and possibly teratogenic if abortion
was not induced. While folic acid deficiency is definitely terato-
genic in animals, it is not yet confirmed in the human. The most
convincing evidence to date seems to be that of Hibbard and
Smithells (1965) in which five times as many mothers with abnormal
formiminoglutamic acid excretion gave birth to infants with severe
malformations, primarily of the CNS, as did mothers with normal
excretion levels.

While the infant may develop folate deficiency in the second
six months of life as a result of dietary deficiency, the low-
birth-weight, preterm baby may develop overt megaloblastic anemia
in the first six months of life. In the latter, no associated
causative factors, such as infection, general malnutrition or ab-
normally low folate diet, may be necessary (Hoffbrand, 1970;

Dallman, 1974).

During lactation, folate is excreted at a level of 50 μg/
liter breast milk (WHO, 1972). Metz (1970) has noted that 100 μg
folic acid per day supplementation to lactating women resulted in
a rise in folic acid in breast milk, but the serum folic acid
levels and reticulocyte counts remained the same. It was con-
cluded that folic acid preferentially goes to the milk secretion.

Impaired Absorption

Absorption of folic acid occurs in the proximal jejunum.
Butterworth et al. (1969) demonstrated that the mucosal cell
accepts only monoglutamate across the cell membrane. That the
brush border converts polyglutamates to monoglutamates by means of
conjugase was suggested by Rosenberg et al. (1969). Beck (1977c)
has pointed out, however, that the assay microorganism used,
L. casei, also utilizes diglutamates and triglutamates.

In studies on isolated intestinal segments of the dog, Baugh
et al. (1971) found pteroylmono- and di-glutamates crossed the in-
testinal mucosa intact. Reduction and methylation were not re-
quired. Rosenberg (1975, 1977) has expanded on the hypothesis
proposed by Rosenberg and Godwin (1971). In this scheme, intesti-
nal enzymes hydrolyze heptaglutamate to monoglutamate. The overall
rate of transport into the mesenteric circulation is controlled by
the movement of monoglutamate. Under appropriate conditions, the
monoglutamate is reduced and methylated in the intestinal cell and
appears in the circulation as methyltetrahydrofolate.

These conflicting results are possibly related to variable
doses of folate, possible presence of folate inhibitors, and the
form of the glutamates (Baker and DeMaeyer, 1979). It is likely
that absorption and transport systems may be resolved by study of
specific genetic defects (Rosenberg, 1975).

Three reports have presented a specific congenital defect in
gastrointestinal absorption of folates (Luhby et al., 1961;
Lanzkowsky et al., 1969; Santiago-Borrero et al., 1973). The case
of Luhby et al. responded to high doses of folic acid orally.
That of Lanzkowsky et al., a product of consanguineous parents, had
associated mental retardation and calcification of the basal
ganglia with absent folate in the CSF. Santini et al. (1973) have
presented a follow-up of the case documented by Santiago-Borrero
et al. (1973).

Active transport mechanism across a gradient has been suggested
by the work of Hepner et al. (1968) and reviewed by Cooper (1977).
Hoffbrand et al. (1977) pointed out that folate conjugase is local-
ized in the cell lysozomes and may exist in many forms (possibly

isozymes) in human tissue. A recent report of 2332 patients rou-
tinely admitted to one medical center (Magnus, 1977) noted elevated
plasma conjugase activities in patients with metastatic cancer,
with active liver disease and in those receiving phenytoin. Normal
activities were found in pernicious anemia patients. Low activities
accompanied diagnoses of wide-spread malignancies, hepatic coma,
aplastic anemia, diabetes mellitus, and severe malnutrition. It
was suggested that plasma conjugase may be of value in the diagnosis
or monitoring of certain diseases.

Development of the triple lumen tube to examine intestinal
perfusion offers possibilities for study of transport mechanisms.
In one study, using this technique, glucose was found to enhance
folic acid absorption (Gerson et al., 1971). In other studies,
oral folic acid, but not intramuscular folic acid or oral vitamin
B$_{12}$, increased jejunal glycolytic enzyme activity. There was no
effect on disaccharidase activity.

Folic acid metabolites are primarily excreted by the urine and
in the bile. A small amount is excreted in the feces. In malab-
sorption syndromes folic acid depletion may be hastened by means of
biliary loss with little reabsorption of folic acid.

Recognition of folate binders in milk, liver, kidney, intesti-
nal epithelial cells, leukemic granulocytes, and folate binders
from pregnant women or women taking oral contraceptive agents may
be of importance in conservation of folic acid (Rothenberg et al.,
1977).

Malabsorption Syndromes

Malabsorption of folic acid may occur with any lesion which
interferes with the absorptive mucosal surface of the jejunum by
means of reducing the available number and surface area of the
microvilli, by impairment of the action of conjugase, by presence
of folic acid antagonists, by presence of bacteria or infection, by
competition by intestinal bacteria with reduction of available
folate, by folate binders, or by possible imbalance of nutrients
presented to the jejunum, or to unknown factors.

Where malabsorption is known to exist by clinical evidence,
Beck (1977c) suggests the serum folate be assayed to determine
folic acid deficiency. If the serum folate is low, additional tests
are unnecessary to establish folic acid deficiency. In certain
cases where absorption of D-xylose is normal (the test which is
most frequently abnormal in intestinal malabsorption) further
studies as suggested by Beck (1977c) may be indicated: microbiolo-
gic or isotopic procedures to measure the serum, urine, or fecal
content of folic acid following a test dose of the vitamin.

Interference with the absorptive surface of the jejunal micro-
villi is seen in tropical sprue, non-tropical sprue, jejunal
atrophy, regional enteritis, lymphoma of the small bowel, Whipple's
disease, diabetes mellitus, scleroderma, amyloidosis, and after
resection or bypass of small bowel as utilized in treating intrac-
table obesity. (Refer to Table I).

Manson in the 1880's first described and named the condition,
sprue. It manifests as a wasting disease accompanied by the sympto-
matology of megalobastic anemia and steatorrhea in persons living
in tropical countries. Sometimes there is delayed onset months
after migration from the tropics. The geographic predilection for
certain endemic areas, eg., Puerto Rico, Southern India, and other
areas, and the less common occurrence in other tropical areas is
not understood. Krumdieck et al. (1977) noted that diets high in
beans and pulses, such as used in Puerto Rico, contain naturally
occurring conjugase inhibitors. Plasma conjugase activity de-
creased by four fold within 20 minutes after ingestion of red
kidney beans in the subjects studied. Further investigation to
determine the significance of this finding is needed. Extensive
reviews of the literature (Gardner, 1958; Klipstein, 1968; Chanarin,
1969; Baker and Mathan, 1968; Gorback et al., 1970; Baker, 1972;
Klipstein, 1972; Butterworth et al., 1974; Corcino et al., 1975)
have examined etiologic possibilities.

Corcino et al. (1976) studied six Puerto Ricans with tropical
sprue. Manifestations included steatorrhea, vitamin B_{12} and folic
acid deficiencies, impaired D-xylose absorption, and jejunal histo-
logical abnormalities. From studies done before and after treat-
ment with vitamin B_{12} and folic acid, they concluded that folate
conjugase deficiency was not the cause, as the activity of conju-
gase was greater prior to therapy. In addition, there was no im-
pairment of ability to convert polyglutamates to lower glutamic
acid residues. Disaccharidases were elevated after therapy. They
concluded the defect was a failure to transport folate across the
mucosal cell.

Klipstein et al. (1973) have explained the lesion in part as
a result of depressed gastric juice secretion with resultant in-
ability to maintain sterility of the normal small bowel. Bhat
et al. (1972) found, on peroral intubation and examination of stool
bacteria, a correlation of severity of histological lesions and
the number of bacteria. No correlation with response to treatment
was noted. Klipstein (1972) emphasized the perpetuation of the
gastrointestinal lesion by increased mucosal cell turnover resulting
in increased folate requirements and compromised absorptive ability.

Butterworth et al. (1974) proposed the theory that an im-
balance between "conjugated" or polyglutamate forms and the "free"
or monoglutamate forms of folate occurs in the small bowel

epithelium and other tissues with high cell proliferation rates.

Most workers agree that morphological lesions continue for a long period of time after treatment is begun with folate. Swanson et al. (1966) found evidence of changes up to a year. Others (Rickles et al, 1972) noted the relapsing nature of the disease or persistence of the lesions, even with the addition of antibiotic therapy which is thought to be curative in some cases.

Manifesting a similar symptomatology, non-tropical sprue (idiopathic steatorrhea, gluten-induced enteropathy, or coeliac disease) also is of unknown etiology. Serum folate concentrations are low; steatorrhea is present with fat droplets in the stool; megaloblastic anemia may or may not be present; atrophy of jejunal villi is present; symptomatic and laboratory response occurs with a diet free of gluten.

While the etiology is unknown, gluten-induced enteropathy has been demonstrated to be associated with abnormal immunological findings. Mann et al. (1970) found immunoglobulin M significantly diminished in 12 patients. An additional patient had a selected serum and exocrine immunoglobulin A deficiency. Cooper et al. (1978) found 63 of 314 patients with coeliac disease had a total of 75 identified immunological disorders. Of interest is the implied association of zinc deficiency with small bowel malabsorption, protein-calorie malnutrition, and decreased cellular mediated immunity (Nutrition Reviews, 1979).

Impaired intestinal folate absorption occurs also in alcoholics. In addition, hematopoiesis was suppressed in alcoholic patients by a possible toxic action even when folic acid or folinic acid was given in large doses (Sullivan and Herbert, 1964). The effect of alcohol on jejunal uptake has been studied extensively (Halsted et al., 1967; Halsted et al., 1971; Halsted et al., 1973). Hermos et al. (1972) found abnormal peroral biopsy specimens of duodenal-jejunal junction in two of three folate deficient alcoholics with demonstration of shortened villi, enlarged crypts, and villous epithelial cells.

Systemic bacterial infection appeared to be a factor in impaired folate absorption (Cook et al., 1974) in Zambian patients, contributing to megaloblastic anemia. The authors advise further study using mono- rather than a non-reduced form of folate which they used to determine absorption.

Anticonvulsant therapy, another cause for impaired folate absorption, may act by interference with absorption but the exact mechanism is unclear. Druskin et al. (1962) considered the drugs interfere with deconjugation of dietary folate, and were able to overcome folate deficiency with 25 µg folic acid daily. Many

workers have demonstrated folate deficiency with anticonvulsant
therapy and its treatment with folic acid. Stebbins and Bertino
(1976) reviewed the several theories of action and have concluded
that the triple lumen perfusion studies of absorption have demon-
strated definite folate inhibition in the presence of phenytoin.
Reynolds (1976), in the same monograph, reviewed the neurological
aspects of folate and cautioned that seizures may be precipitated
in convulsive disorders by folate. One death from severe anemia
has been reported in a patient in whom anticonvulsant-induced
anemia was superimposed upon Hemoglobin H thalassemia disease (a
congenital abnormal hemoglobin with associated hemolysis) and
resulted in terminal heart failure (Pinkhas et al., 1973).

Numerous but conflicting reports have implicated oral contra-
ceptives, especially those containing mestranol, with folic acid
deficiency, low serum folate concentrations, megaloblastic anemia,
or with local cytological changes of the cervix. The latter is
thought to be an end-organ manifestation of conjugase inhibition
(O'Malley and Means, 1974) which has been shown to be reversible
within three weeks by pharmacological doses of folic acid (Whitehead
et al., 1973). While the mechanism is unclear, Streiff (1970b)
demonstrated in patients taking oral contraceptives poor absorption
of food polyglutamates but normal absorption of monoglutamates.
Streiff and Greene (1970) reported inhibition of conjugase activity
in vitro by mestranol. Stephens et al. (1972) were unable to con-
firm in vitro inhibition. Further, no difference in absorption
between control subjects and women taking oral contraceptive agents
was found provided they were saturated with folic acid. Without
presaturation, a significant absorptive reduction occurred in the
patients taking oral contraceptive agents. Recent studies by
daCosta and Rothenberg (1974) have demonstrated a folate binder in
sera of 24 of 51 pregnant women, 8 of 10 on oral contraceptives,
and none of 15 women who were not pregnant nor taking oral contra-
ceptive agents. They attributed these changes to a potentiation
by oral contraceptive agents of inadequate folate intake or folate
need by removal of folate from the body by the folate binder.
Other workers have indicated the effect of oral contraceptives may
be related to borderline diet adequacy of folate (Castrén and Rossi,
1970; Necheles and Snyder, 1970; Pritchard et al., 1971).

Other drugs have been associated with folate deficiency:
antimalarials (Strickland and Kostinas, 1970; Sheehy and Dempsey,
1970), azulfidine (Franklin and Rosenberg, 1973), triamterene
(Corcino et al., 1970), tetracycline (Jones, 1973), methyl-dopa
(Schneerson and Gazzard, 1977), and neomycin (Jacobson et al., 1960).

Folic Acid Requirements in Hemolytic and Other Diseases

Folic Acid requirements are significantly increased in any
disease in which a hemolytic process is associated. These include

sickle cell anemia (Huntsman, 1974), thalassemia (Jandl and Green-
berg, 1959), myeloproliferative disorders (Chanarin, 1970), sidero-
blastic anemias (Mollin and MacGibbon, 1972), and aplastic anemia
(Branda et al., 1978). In the aplastic anemia patient studied by
Branda et al. (1978), a high dose of folic acid, 20 mg per day,
induced a slow erythropoietic response with no further need for
transfusions. The patient continued to have leucopenia and throm-
bocytopenia. This work is important in that family studies de-
monstrated, among four generations of the patient's family, 20
members with histories suggestive of severe hematologic disorders.
The patient's PHA stimulated leucocytes demonstrated marked sup-
pression of [14]C-methyltetrahydrofolate uptake but normal [14]C-thymi-
dine uptake. Uptake of bone marrow cells reacted similarly. Five
other family members tested, including the patient's son, demon-
strated decreased [14]C-methyltetrahydrofolate uptake by stimulated
lymphocytes.

An unexplained lack of responsiveness to folate therapy in
thalassemic patients among Chinese in Hong Kong, even though sub-
normal serum folate levels were noted, has been reported by Tso
(1976). That megaloblastosis due to folic acid deficiency is
uncommon among Chinese has been related to the high level of green
and leafy vegetables in the diet as well as to their cooking
practices.

Folic acid metabolism in renal disease patients has been
studied by Retief et al. (1977) and Colman and Herbert (1974),
Folate binding protein was elevated in the serum of uremic patients,
probably as a result of impaired reabsorption by the tubules.
Serum binders may withhold folic acid from the tissues.

Exfoliative dermatitis is another etiologic cause of increased
requirement of folic acid as Hild (1969) has found losses of folic
acid of 5 μg to 20 μg per day. The folic acid content of the skin
was greater than that in normal skin. Changes of the epidermis have
been noted with vitamin B$_{12}$ deficiency (Gilliam and Cox, 1973).

Folic acid antagonists have been used effectively in treating
neoplastic diseases, acute leukemia in children, choriocarcinoma,
head and neck neoplasias (usually for squamous cell carcinoma), and
in adjuvant chemotherapy in conjunction with radiation therapy,
and with other antimetabolic drugs. Concentration of folic acid in
leukemic cells was demonstrated to be elevated in both acute and,
less so, in chronic leukemia by Swenseid et al. (1951). Folic acid
antagonists inhibit the reduction of dihydrofolate to tetrahydro-
folate. As a consequence, DNA, RNA, and protein synthesis are
inhibited.

The chemistry, enzymology, mechanisms of action and use of
anti-folates have been reviewed in a recent symposium (Bertino, 1971).

More recently, Bertino et al. (1977) have reviewed augmentation of
anti-folate action by metabolic transport inhibitors which prevent
efflux of the antimetabolite from the cells. Another new and
potentially useful possibility for cancer chemotherapy has been
suggested by Huennekens et al. (1976) by alteration of the
methionine synthetase content of cells and thus interfering with
the vitamin B_{12}-folate acid interrelationship (see VITAMIN B_{12}
DEFICIENCY, Biochemical Considerations).

Degradation of folic acid to form pterin-6-aldehyde in cancer
patients has been reported by Halpern et al. (1977). In no
hospital patient tested was the test positive in the absence of
cancer. The significance of this finding remains to be shown.

A major breakthrough in the use of high doses of anti-folate
drugs in cancer chemotherapy was the use of citrovorum factor to
"rescue" the normal cells by selective action (Frei et al., 1975).

Neurological Considerations of Folic Acid Deficiency

In the last decade, a growing body of evidence indicates that
folic acid deficiency results in neurological symptomatology. The
subject has been reviewed by Reynolds (1976).

That anticonvulsants interfere with folic acid metabolism and
absorption is well documented. There is a high correlation of
anticonvulsant medication with low serum folic acid concentrations
and with CSF folate (Reynolds, 1971). A study on marrow cells from
epileptic patients taking anticonvulsants reported no correlation
between the degree of megaloblastosis and the deoxyuridine sup-
pression test (Taguchi et al., 1977). The authors suggested that
anticonvulsants may exert their effect at a stage of DNA synthesis
beyond that at which folate coenzymes function. Treatment of
epileptics with folic acid has resulted in increase in seizure
attacks (Reynolds, 1973) and a return to a normoblastic marrow in
those with megaloblastic marrows (Reynolds, 1968). The mechanism
of action of folate to increase seizure frequency is not known.
Mayersdorf et al. (1971) have found, however, that folate concen-
tration in the region of epileptic foci induced by metallic cobalt
powder is higher than in other regions of the brain cortex.

The mechanism of transport of reduced folate to the CSF is
thought to be by way of the choroid plexus (Spector and Lorenzo,
1975; Bertino et al., 1977; Spector, 1977).

The depression, irritability, and other mental disturbances
reported in experimentally induced folate deficiency by Herbert
(1962a) and noted in diseases accompanied by folate deficiency have
been thought to be of significance in psychiatric illness (Reynolds,
1976). Shulman (1972), however, considers folate disorders in

psychiatric illness to be related to malnutrition, chronic diseases, or to alcohol and other drug use.

It is of significance that one case of well-documented folate deficiency presented with subacute combined system disease, which did not respond either hematologically or neurologically to vitamin B$_{12}$, whereas folic acid administration produced a marked recovery (Pincus et al., 1972).

INBORN ERRORS OF METABOLISM RESULTING IN MEGALOBLASTIC ANEMIA AND MEGALOBLASTIC ANEMIA IN CHILDREN

In 1960, the first case of an absorptive defect of vitamin B$_{12}$ in children was reported by Imerslund (1960). Ten patients from six families were found. Onset ranged from age five to six months up to four years. The findings differed from those of Addisonian pernicious anemia in that the patients had gastric hydrochloric acid secretion after histamine, normal IF activity, and an associated proteinuria. Gräsbeck et al. (1960) proposed a recessive gene inheritance for this condition. It responds promptly to vitamin B$_{12}$ therapy.

Congenital IF deficiency is also a rare disease in which the impaired vitamin B$_{12}$ absorption is correctable by administration of IF. Patients have normal gastric acid secretion, normal gastric mucosa, and no associated endocrinopathy (McIntyre et al., 1965; Miller et al., 1966). Katz et al. (1972) reported malabsorption due to a biologically inert IF.

A second group of pernicious anemia in childhood is that in which gastric acidity is absent; gastric mucosal atrophy is present; and parietal cell and IF antibodies are occasionally present (Farrell et al., 1979). Earlier, Herbert et al. (1964) considered this anemia not to have an autoimmune mechanism. Oliver and Baker (1969) reported a 21 year old man with sudden onset of pernicious anemia in whom hypothyroidism had been diagnosed at age six years. The patient's father and aunt had Addisonian pernicious anemia. Gastric and thyroid antibodies were demonstrated in all three and also in the patient's mother.

Methylmalonic aciduria responsive to vitamin B$_{12}$ has been described prenatally at midterm by Ampola et al. (1975). Treatment of the mother with large doses of vitamin B$_{12}$ resulted in successful termination of the pregnancy nine weeks after start of therapy. The enzymatic block was found to be a deficiency of 5'deoxyadenosylcobalamin. Prenatal diagnosis was suspected as the mother had had a first child with methylmalonic aciduria who died at three months of age. The child reported was maintained subsequently with a low protein diet.

By studies of the synthesis of 5'-deoxyadenosylcobalamin in
fibroblast extracts from patients with inherited methylmalonic
acidemia, Mahoney et al. (1975) showed biochemical heterogeneity
in the phenotype with deficient accumulation of adenosylcobalamin.
Thus, genetic heterogeneity is implied by their finding of at
least two mutant classes in this phenotype.

Linnell et al. (1976) used two dimensional chromatobioauto-
graphy to study cobalamin metabolism in cultured fibroblasts and
skin biopsies taken from normal control subjects and from patients
with methylmalonic aciduria. The latter fell into three classifi-
cations of abnormality: Group A, defective methylmalonyl-CoA
mutase apoenzyme, but no abnormality of cobalamin metabolism;
Group B, failure to accumulate adenosylcobalamin; and Group C,
failure to accumulate either adenosylcobalamin or methylcobalamin.

Congenital disorders of vitamin B_{12} transport and storage have
been reported and reviewed by Cooper (1976). TC II deficiences
lead to severe megaloblastic anemia, neurological deterioration and
infections (Hakami et al., 1971; Gimpert et al., 1975; Burman
et al., 1979). An autosomal recessive disorder, TC II deficiency
is responsive to massive doses of vitamin B_{12}. TC R-type binder
deficiency has been reported but has no known clinical manifesta-
ions (Carmel and Herbert, 1969).

Inborn errors of folate metabolism have been classified
according to whether the defect is of uptake, interconversion, or
utilization of folates (Erbe, 1975). These are: congenital malab-
sorption of folate; interconversion defects: dihydrofolate reduc-
tase deficiency, methenyl-tetrahydrofolate cyclohydrolase deficiency,
methylene-tetrahydrofolate reductase deficiency; and defective
folate utilization: 5-methyltetrahydrofolate homocysteine methyl-
transferase deficiency, and glutamate formiminotransferase
deficiency.

Erbe (1975) also reviewed related inborn errors affecting
folate metabolism: inborn errors of vitamin B_{12} metabolism,
methionine adenosyl transferase deficiency, dihydropteridine re-
ductase deficiency, non-ketotic hyperglycinemia, Lesch-Nyhan
syndrome, orotic aciduria, and cystathionine synthetase deficiency.

Another unusual case presentation has recently reported the
prenatal diagnosis of a deficiency of propionyl-CoA carboxylase
(Sweetman et al., 1979). This enzyme mediates propionic acid
metabolism, the immediate precursor of methylmalonic acid.
Rosenberg (1969) reviewed vitamin-dependent aminoacidopathies.

Additional errors of metabolism have been mentioned in pre-
ceding sections.

While these defects are rare, more may be diagnosed and effectively treated as techniques for in utero diagnosis become available. At the present time, Milunsky (1976) includes the following in which this is possible and which are associated with defective vitamin B$_{12}$ or folate metabolism: methylmalonic aciduria responsive to vitamin B$_{12}$, vitamin B$_{12}$ metabolic defect, and methylene tetrahydrofolate reductase deficiency. Another important aspect of the inborn errors of metabolism may be the opportunity to define more clearly the basic cellular and subcellular mechanisms which are not clearly understood at present.

SUMMARY

This chapter has reviewed some of the remarkable history in the advances in understanding of vitamin B$_{12}$ and folic acid. Newer knowledge in the mechanisms of action and their interrelationships through the disciplines of nutrition, hematology, medicine, biochemistry, physics, bacteriology and physiology continues to progress.

In the past decade, development of new techniques has led to the identification of the vitamin B$_{12}$ transport proteins, to a better knowledge of how the vitamin enters the cell, the enzymatic mechanisms through which the two vitamins interrelate, and the genetic basis by which certain of the deficiencies develop.

The study of the manifestations of the impairment of DNA synthesis, which lack of vitamin B$_{12}$ and folic acid causes, continues to lead to solutions which have implication for wider application such as in the study and treatment of cancer, genetic defects, and disorders of immune mechanisms.

ACKNOWLEDGEMENTS

Dr. Ralph Carmel kindly supplied references for work on the megaloblastic effect of anesthetic gases.

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TRACE METALS IN HEALTH AND DISEASE

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INTRODUCTION

Trace elements often have a bimodal or even a trimodal effect. Severe deficiency of certain trace metals can result in death or severe crippling of an animal or in birth defects of the newborn. The next level of intake is the nutritional level where chronic deficiency over a lifetime may cause major diseases such as cancer and heart disease. The next level of intake is the toxic level which may result in severe crippling or death of the animal. The primary area of concern in this chapter will be the nutritional intake level and the deficiency level and their relationship to animal or human disease. The trace elements described in this chapter are: chromium; cobalt; copper; fluorine; iodine; iron; manganese; molybdenum; nickel; silicon; selenium; tin; vanadium; and zinc.

CHROMIUM

Glucose Metabolism

The most prominent feature of chromium deficiency in rats (Schwarz and Mertz, 1961) and other animals (Davidson and Blackwell, 1968) is impairment of glucose tolerance. After a few weeks on a torula yeast diet, glucose removal rates declined almost 50%. The decline can be reversed quickly by one oral dose of 20 μg or an intravenous dose of 0.25 μg/100 g body weight of trivalent chromium (Mertz et al., 1961; Mertz et al., 1965). Chromium deficiency in a more severe degree leads to a syndrome like mild diabetes mellitus, including glycosuria and fasting hyperglycemia.

Since 1966, evidence for a human nutritional requirement for chromium has been looked at in patients with an impairment of glucose tolerance including "maturity onset" diabetes, middle-aged and elderly subjects with impaired glucose tolerance, and infants with protein-calorie malnutrition. In the first therapeutic trial (Glinsmann and Mertz, 1966) four of six maturity onset diabetics improved after administration of 180 to 1000 μg of chromium as $CrCl_3$ for periods of 7 to 13 weeks. In another study (Sherman et al., 1968) treated 10 adult diabetics with 150 μg daily for 16 weeks and observed no improvement of glucose tolerance. In another experiment 4 of 12 diabetics treated with 1 mg chromium each day for six months had improved glucose tolerance, but 16 treated for a shorter time period with 150 μg chromium daily did not improve (Schroeder, 1968). In 10 elderly patients (over 70) treated up to four months with 150 μg of chromium, glucose tolerance was restored to normal in four (Levin et al., 1968). Half of a group of middle-aged subjects treated with 150 μg daily for 6 months had a marked improvement (Hopkins and Price, 1968). Benjanuvatra and Bennien (1975) have measured hair chromium analyses for 28 subjects with adult-onset diabetes mellitus and 28 nondiabetic control subjects from Bangkok, Thailand. Hair chromium concentrations were significantly lower in the diabetic than in the control group.

Davidson and Burt (1973) have suggested that the "diabetogenic" effect of pregnancy is related to a significantly lower concentration of plasma chromium of pregnant women. The "diabetogenic" effect of pregnancy is characterized by impairment of peripheral glucose metabolism, decreased glucose tolerance, and an exaggerated insulin excretion in response to glucose challenge.

Morgan (1972) has measured liver chromium content from postmortem examinations in elderly patients with diabetes, with ischemic heart disease, with hypertensive cardiovascular disease, and without disease. The results in μg/g were controls 12.7; arteriosclerotic 9.96; hypertensive 10.2; and diabetics 8.59. The diabetic group was significantly decreased in regard to the control population (p = 0.05). Serum chromium has been related (Newman et al., 1978) to angiographically determined coronary artery disease. Human aortas sampled from 17 adults where there is little advanced atheromatous plaque formation contain higher concentrations of chromium than do aortas from 15 adults in which atheromatosis is prevalent.

In a study on a patient with long term total parenteral nutrition Jeejeebhoy et al., (1977) have reported that a chromium deficiency in man, causes: (1) glucose intolerance, (2) inability to utilize glucose for energy, (3) neuropathy with normal insulin concentrations, (4) high free fatty acid concentrations and low respiratory quotient and, (5) abnormalities of nitrogen metabolism.

Lipid Metabolism

Serum cholesterol concentrations in rats were lowered by addition of chromium to a low-chromium diet and chromium inhibited the tendency of these cholesterol concentrations to increase with age. Male rats fed 1 μg Cr/ml and females fed 5 μg/ml in the drinking water had significantly depressed serum cholesterol (Schroeder et al., 1962; Schroeder and Balassa, 1965; Schroeder, 1969). Brown sugar, which contains chromium, also lowered serum cholesterol concentrations, but cholesterol was elevated and increased with age in the rats receiving white sugar, which is low in chromium (Schroeder, 1969; Staub et al., 1969). Significant decrease of serum cholesterol was seen in institutionalized patients fed 2 mg Cr for 5 months, but other patients treated similarly showed no response (Schroeder, 1968).

Significantly lower (2%) incidence of spontaneous plaques were observed in the chromium-fed animals than chromium-deficient animals (19%) (Schroeder and Balassa, 1965). Decreased amounts of stainable lipids and of fluorescent material in the aorta were observed. Similar observations have been made in man in that diabetes is associated with an increased incidence of vascular lesions (Goldenberg and Blumenthal, 1964). Curran (1954) has observed that trivalent chromium enhances the incorporation of acetate into the formation of cholesterol and fatty acids. Mertz et al. (1965) have observed that chromium plus insulin significantly increases glucose uptake and incorporation of glucose into epidedymal fat in chromium-deficient rats.

Protein Synthesis

Diets deficient in chromium and protein impair the rats' capacity to incorporate α-amino isobutyric acid, glycine, serine and methionine into heart protein (Roginski and Mertz, 1967a; Roginski and Mertz, 1967b). No chromium effect was observed with lysine, phenylalanine or a mixture of 10 other amino acids. Insulin in vivo enhanced the cell transport of an amino acid analog to a greater degree in rats fed a low-protein, chromium-supplemented diet than it did in Cr-deficient controls (Roginski and Mertz, 1969).

COBALT

Cobalt deficiency is not know in man. However, cobalt is an essential component of vitamin B-12 which normally occurs in the serum in the range of 150-900 pg/ml. Concentrations of serum B-12 from 0-150 pg/ml are considered low. The following is a general discussion about B-12. Far greater details are contained in this monograph in the chapter by Steinkamp.

Vitamin B-12 deficiency secondary to inadequate intake is rare

in the western world except among Seventh Day Adventists, food
faddists and infants breast-fed by vegetarian mothers. B-12
deficiency occurs only after a prolonged period of inadequate
intake since such subjects secrete adequate quantities of gastric
intrinsic factor (GIF) and thus continue to reabsorb B-12 that is
secreted into the small intestine via bile. Pernicious anemia (PA)
patients develop a deficiency after a shorter depletion period be-
cause they are unable to reabsorb endogenous vitamin B-12 into the
bile because of the lack of GIF. When normal gastric function
exists, the release of vitamin B-12 from dietary protein does not
restrict the rate of B-12 absorption. However, in gastric atrophy
or after subtotal gastrectomy, acid-peptic digestion is depressed
and the release of free vitamin B-12 from dietary sources may be
impaired.

Vitamin B-12 may fail to attach to GIF. The most common
cause is the lack of GIF associated with Addisonian pernicious
anemia. GIF secretion is markedly depressed, as part of the gener-
alized atrophy of the acid pepsin portion of the stomach. Patients
with idiopathic gastric atrophy or after gastric surgery may have
depressed secretion of GIF in the basal state. GIF antibodies may
also occur, but are rarely found in diseases other than pernicious
anemia. A few patients with adrenal insufficiency, diabetes mel-
litus, hypothyroidism, and thyrotoxicosis have serum GIF antibodies.

Another group of malabsorptive diseases are associated with
the passage of the GIF-vitamin B-12 complex down the small intestine.
The blind loop syndrome defect appears to be associated with bac-
terial overgrowth. The absorptive defect appears to result from
the intraluminal competition of the bacteria with the host for
dietary B-12. The mechanism of B-12 malabsorption after infesta-
tion with the fish tapeworm Diphyllobothrium latum or patients with
pancreatic exocrine insufficiency has not been explained. A damaged
pancreas may not be able to buffer the gastric acid output of the
stomach and would impair B-12 absorption.

Certain B-12 malabsorption diseases are associated with the
passage of B-12 through the ileal epithelial cell. Children with
familial selective vitamin B-12 malabsorption (Imerslund or Gras-
beck syndrome) may lack the specific ileal receptors for the GIF-
vitamin B-12 complex. Malabsorptive disease may be associated with
the exit of B-12 from the epithelial cell. Transcobalamin-II
deficiency has been described. A number of therapeutic agents have
been shown to interfere with the absorption of B-12 including al-
cohol, p-aminosalicylic acid, colchicine, metformin and neomycin.

Serum B-12 concentrations well over 1000 pg/ml of serum suggest
either liver disease or a myeloproliferative disorder (such as
granulocytic leukemia, myeloid metaplasia or polycythemia vera;
values above 4000 pg/ml are unusual in liver disease but common in

myeloproliferative disorders.

COPPER

Deficiency Anemia

Malnourished infants with anemia and neutropenia caused by copper deficiency have been reported (Cordano et al., 1964; Cordano et al., 1966; Al-Rashid and Spangler, 1971; Karpel and Peden, 1972; Graham and Cordano, 1969). In general, copper is widely distributed in foods making it virtually impossible for severe copper deficiency to develop on a nutritional basis in the adult. Dunlop (1974) has reported a copper deficiency in an adult woman and an adolescent girl who had experienced extensive bowel surgery and received long-term parenteral hyperalimentation. Another case was reported in a 56-year old woman on intravenous hyperalimentation (Dunlop, 1974). Megaloblastoid changes in the bone marrow were also seen in some of the infants with copper deficiency (Cordano et al., 1964; Al-Rashid and Spangler, 1971). The megaloblastic changes were corrected by copper, which implies an effect on RNA/DNA synthesis in the de nova pathway of deoxyuridine to thymine. The neutropenia, which is thought to be the most constant sign of copper deficiency, was reversed by copper therapy. Neutropenia has been reported in all of the copper-deficient infants (Cordano et al., 1964; Cordano et al., 1966; Al-Rashid and Spangler, 1974; Karpel and Peden, 1972; Graham and Cordano, 1969). Neutropenia has also been reported in copper-deficient swine (Wintrobe et al., 1953).

Cardiovascular Disorders

Bennetts and coworkers (Bennets and Hall, 1939; Bennetts et al., 1941; Bennetts et al., 1942) first noticed cardiac lesions in western Australian cattle suffering from "falling disease". In these cattle there was an atrophy of the myocardium with replacement fibrosis with dense collagenous tissue. Death resulted due to cardiac failure after mild exercise or excitement. In other animals such as sheep, horses and pigs the myocardium is rarely affected. In chicks (O-Dell et al., 1961; Carlton and Henderson, 1963; Simpson and Harms, 1964; Hunt et al., 1970), pigs (Carnes et al., 1961; Coulson and Carnes, 1963; Shields et al., 1961) guinea pigs (Everson et al., 1967) and rats (Kelly et al., 1974) on copper-deficient diets, ruptures of the coronary and pulmonary arteries have been observed. The tensile strength of the aorta in these species was markedly reduced and the myocardium was abnormally friable. A derangement of the elastic membranes of the aorta was evident. Similar studies have not been done on human patients with aneurysms, but such a study would be of interest. Copper deficiency in rats resulted in a 129% increase in serum cholesterol (Allen and Klevay, 1978) and a copper deficiency

in rats over 3 generations produced a 56% decrease in myelin (Zimmerman et al., 1976). Klevay and Forebush (1976) have postulated that the zinc/copper ratio of human dietary intakes is related to heart disease.

Wilson's Disease

Wilson's disease or hepatolenticular degeneration is an inherited autosomal recessive disorder, described first by Wilson (1912). This disease affects mostly the liver, basal ganglia of the brain, cornea, and the kidneys. Chronic degeneration of the basal nuclei is associated with degeneration of the hepatic parenchyma, which leads to eventual cirrhosis. This disease is progressive and ultimately fatal. The most consistent feature has been severe dimunition or occasional absence of serum ceruloplasmin. Two major hypotheses have been advanced to account for the pathological findings of Wilson's disease. Hypothesis A: An abnormal gene suppresses the synthesis of normal ceruloplasmin (Bearn, 1953; Scheinberg and Gitlin, 1952; Cartwright et al., 1954; Earl et al., 1954). As a consequence of ceruloplasmin deficiency, copper absorption from the gut is accelerated leading to excessive copper being deposited in the tissues, resulting in structural damage and functional impairment in the organs involved. A second hypothesis that an abnormal protein, presumably determined by the mutant gene, has an enhanced affinity for copper. This deprives ceruloplasmin of its copper, blocking its formation, hence the deficiency (Iber et al., 1954; Uzman et al., 1956). Neither hypothesis has been proven. The biochemical defect could also be related to a decreased cytochrome oxidase activity. Individuals with Wilson's disease have extremely low activities of leucocyte cytochrome oxidase, comparable to the low levels of ceruloplasmin in their serum (Shokeir and Shreffler, 1969). Carriers of Wilson's disease display intermediate activities of cytochrome oxidase in the leucocytes and ceruloplasmin in the serum.

Torsion dystony is somewhat similar to Wilson's disease and is also a copper metabolic defect (Herishanu and Loewinger, 1972). This pathological and clinical condition is also called dystonia lenticularis.

Menkes Kinky Hair Syndrome

Menkes syndrome (Trichopoliodystrophy) is a progressive brain disease of male infants, which usually causes death before 3 years of age and which is characterized by severely retarded growth and development, "kinky hair" (pili torti), hypothermia, scorbutic bone changes, arterial tortuosity and cerebral gliosis with cystic degeneration (Menkes, 1972). Alterations of free sulfhydryl groups of hair may result in its tendency to twist (Danks et al., 1972). Menkes syndrome is associated with profound hypocupremia,

hypoceruloplasminemia, and diminished concentrations of copper in hair (Danks et al., 1972a; Danks et al., 1972b; Danks et al., 1973; Singh and Bresnan, 1973). Copper deficiency may be related to the changes in the elastic fibers of arterial walls and scorbutic bone deformities (Danks et al., 1972a; Danks et al., 1972b). There is also a defect in intestinal copper absorption which could be caused by either a disturbance of the intracellular transport of copper in the duodenal mucosa or an impairment of copper transport across the serosal cell membrane (Danks et al, 1973). Beneficial results may be derived from parenteral administration of copper (Danks et al., 1972a; Danks et al., 1972b; Danks et al., 1973). Screening of infants for Menkes syndrome as well as Wilson's disease by analyses of serum copper could become a major application of clinical pathology laboratories (Sunderman, 1974).

Hodgkins Disease

     Measurements of serum copper provide valuable laboratory information in monitoring relapse in patients with Hodgkin's lymphoma because there is a good correlation between the concentration of serum copper and the clinical activity of the lymphoma (Hrgovcic et al., 1968; Warren et al., 1969). Hyperceruloplasminemia occurs as a manifestation of the "acute phase reaction" during active Hodgkins disease. Serum concentrations of copper may help guide the physician in anticipating the clinical response to treatment in patients with Hodgkins disease. Increased serum copper concentration is not specific for Hodgkins disease activity, but can occur with increased estrogen and with various nonmalignant processes including heart disease and anemia. Wilimas et al. (1978) have found serum copper concentrations to be unreliable as a diagnostic aid in the individual child. A large number of patients had elevated serum copper concentrations and were free of the disease. In addition, copper was elevated in the serum of 19 patients with sarcomas (Fisher et al., 1976). Copper was also elevated in the serum of 56% of patients with schizophrenia (Chugh et al., 1973). Patients with psoriasis tend to have higher than normal serum copper concentrations (Lipkin et al., 1962; Kekki et al., 1966; Molokhia and Portnoy, 1970).

FLUORINE

Dental Caries

     A relationship between dental caries and increasing fluoride concentration was first shown by Dean (1942). In a study of 7,257 children, 12-14 years old, caries decreased in permanent teeth as the drinking water fluoride concentration increased above 1.3 ppm. In many countries an inverse relationship has been observed between naturally present fluoride in the water and dental caries (Forrest et al., 1-51; McKay, 1948; Russell and Elvove, 1951).

The water supplies of hundreds of communities have been treated
with sodium fluoride or fluorosilicate at 0.8 to 1.2 ppm. When
these communities were compared to adjacent communities without
fluoridation, the value of fluoridation was observed (Arnold,
1957; Naylor, 1969). Topical applications of fluorides have
achieved some success (Harris, 1959; Knutson and Armstrong, 1943;
1945; 1946; 1947; Jordan et al., 1958).

Osteoporosis and Aortic Calcification

Osteoporosis is a disease of the elderly characterized by a
decrease in bone density often accompanied by collapsed vertebrae;
large amounts of fluoride improve osteoporosis in these patients
(Aeschlimann et al., 1966; Cass et al., 1966; Cohen and Gardner,
1966; Purves, 1962; Rich, et al., 1964). In two comparative studies
Leone et al., (1960 have found less osteoporosis in a high fluoride
area of Texas (8 ppm in water) than in a low-fluoride area of
Massachusetts (0.09 ppm in the water) and Bernstein et al. (1966)
have found similar results in a comparative study of high- and
low- flouride areas of North Dakota.

Leone (1964) has compared the mortality of a high and low-
fluoride area of Texas. The low fluoride area had a much greater
incidence of heart attacks. Calcification of the aorta was much
greater in the low-fluoride area.

Cancer Controversy

There has been a good deal of interest in a purported relation
between community water fluoridation and cancer mortality. Burk
(1975) and Yiamouyiannis (1975; 1977) reported on cancer mortality
trends in 20 United States cities, half of which fluoridated their
water supplies during 1952-1956. In 1950 the crude mortality rates
for cities with fluoridated water supplies were very nearly equal
to the rates in the cities with non-fluoridated supplies. In 1970
there was a differential of some 20 deaths per 100,000 persons,
the higher rates being in the cities with fluoridated water. Re-
analysis of this data pertaining to these cities (Doll and Kinlen,
1977; Taves, 1977) and a similar study of cancer mortality in
selected counties in the United States with fluoridated and non-
fluoridated water systems (Hoover et al., 1976) did not support
the data of Burk (1975) and Yiamouyiannis (1975).

IODINE

Goiter

The first large scale trial on iodine deficiency and goiter
in man was carried out in the schools of Ohio between 1916 and
1920. Similar prophylactic measures were soon initiated in

goitrous areas in other countries with such success that iodized
salt soon became a widely recognized form of control. Nonetheless,
the actual number of goitrous individuals in the world is estimated
in 1960 at close to 200 million (Kelly and Snedden, 1960). Women
and children are more affected than adult males (Malamos et al.,
1966). In the United States, Switzerland and New Zealand, the
intensity of the disease declined markedly as a result of the use
of iodized salt. In many Latin American countries, despite the
simplicity of needed control measures, endemic goiter remains a
serious public health problem, associated often with cretinism,
feeblmindedness and deaf-mutism (Scrimshaw, 1960).

    The healthy human adult probably contains a total of 10 to
20 mg iodine, of which a high proportion, 70-80%, is concentrated
in the thyroid gland which is only about 0.05% of the body mass.
Iodine occurs as organically bound iodine and as inorganic iodide.
The latter occurs in most non-thyroid tissues in very low concen-
trations, of the order of 1 to 2 µg/100 g.

Thyroid Function

    The normal range of serum protein-bound iodine in adult man
has been placed at 4 to 8 or 3 to 7.5 µg/100 ml with a mean close
to 5 to 6 µg (Grossmann and Grossmann, 1955; Hallman et al., 1951;
Rapport and Curtis, 1950; Wayne et al., 1964). Increased serum
PBI values are highly characteristic of hyperthyroidism and de-
creased values of hypothyroidism in man. However, under a variety
of conditions a discrepancy between the serum PBI and the patient's
clinical thyrometabolic state was observed. The most common cause
of spurious elevation of the PBI is exposure to a variety of iodine-
containing drugs and radiographic contrast media and estrogen ther-
apy. Development of T_4 tests (3,5,3,5,-Tetraiodothyronine), T_3
uptake (3,5,3-Triiodothyronine) and free thyroxine index has re-
sulted in better diagnostic accuracy and has widely supplemented
the PBI procedure.

IRON

Deficiency Anemia

    Human iron deficiency is clinically characterized by listless-
ness and fatigue, palpitation on exertion, sore tongue on occasion,
koilonychia, and angular dysphagia (Darby, 1951). In children,
anorexia, depressed growth, and decreased resistance to infection
are additionally commonly observed. In iron-deficient anemia ab-
normalities of the gastro-intestinal tract, including superficial
gastritis and achlorhydria have been observed (Hawksley et al.,
1934; Lees and Rosenthal, 1958). Anemia due to iron-deficiency
is much more common in women than in men because women of fertile
age are subject to additional iron losses in menstruation,

pregnancy and lactation. The incidence of iron deficiency in women
has been reported as 20-25% in different English and Swedish studies
(Laufberger, 1937; Rybo, 1966). Among pregnant women in the United
States, iron-deficiency anemia frequencies ranging from 15% to 50%
have been observed (Allaire and Campagna, 1961; Pritchard and Hung,
1958). In adult men and postmenopausal women the principal cause
of anemia is chronic bleeding due to infections, malignancy, bleed-
ing ulcers, and hookworm infestation. Economic disadvantage (Both-
well and Finch, 1962; Mukherjee and Mukherjee, 1953), infection,
vegetarian-type diets (Hussain et al., 1967), and excessive sweat-
ing can also lead to iron-deficiency anemia.

During the suckling period, both young animals and humans can
also become iron deficient (von Bunge, 1899; Ezekiel, 1967). In
the United States, iron-deficiency anemia may range as low as 8%
to as high as 64% (Beal et al., 1962; Sturgeon, 1956). Between
4 and 24 months of age is when iron-deficiency anemia occurs most
frequently. There is a regular need for supplementation with
medicinal iron or iron-fortified foods (Beal et al., 1962; Stur-
geon, 1956; Farquhar, 1963).

States with Increased Serum Iron

Increases in serum or plasma iron are brought about by several
conditions: increased red-cell destruction (decreased survival of
red cells, hemolytic anemia), in cases of decreased utilization
(decreased blood formation as in pyridoxine deficiency or in lead
poisoning), in situations in which release of iron occurs (release
of ferritin in necrotic hepatitis), in states where iron storage
is defective (as in pernicious anemia), and in conditions in which
there is an increased rate of absorption (e.g., hemosiderosis and
homochromatosis).

States with Decreased Serum Iron

Decreases in serum or plasma iron are generally due to a de-
ficiency in the total amount of iron present in the body which
may be caused by a lack of sufficient intake or absorption of iron
(nephrosis or chronic blood loss) or by an increased demand of the
body stores (pregnancy). Diminished iron levels may also be
caused by a decreased release of iron from body stores (reticulo-
endothelial system) as seen in infections or abscesses.

States with Altered Iron-Binding Capacity

An increased production of transferrin may be related to in-
creases in the total iron-binding capacity of serum. Increases
in the total iron-binding capacity may also be caused by an in-
creased release of ferritin, as in hepatocellular necrosis. De-
creases in the total iron-binding capacity may be caused by a

deficiency in ferritin, as found in cirrhosis and hemochromatosis, or as a result of an excessive loss of protein (transferrin) occurs in nephrosis.

MANGANESE

Deficiency

Human requirements for manganese are not known, and there was even a doubt that a deficiency could occur in man until Doisy (1973) recognized the first case in a volunteer undergoing a study of vitamin K deficiency in a metabolic ward. Failure to add Mn to a purified diet mixture resulted in weight loss, transient dermatitis, nausea, and slow growth of hair and beard with changes in hair color, and biochemically there was striking hypocholesterolemia.

Lipid Metabolism

Liver and bone fat are reduced in manganese- and choline-supplemented rats, and back fat is reduced in pigs (Plumlee et al., 1954; 1956). Because manganous ion is a necessary cofactor for the conversion of mevalonic acid to squalene by mevalonic kinase (Amdur et al., 1957), manganese stimulates the hepatic synthesis of cholesterol and fatty acids in rats (Curran, 1954).

Carbohydrate Metabolism

Manganese may be important in gluconeogenesis through its presence in the metalloenzyme pyruvate decarboxylase. Aplasia or marked hypoplasia of guinea pig pancreas occurred in Mn-deficient animals (Everson and Shrader, 1968; Shrader and Everson, 1968), and decreased the number of pancreatic islets. A Mn-deficient guinea pig shows a decreased capacity to utilize glucose and exhibits a diabetic-like curve in response to glucose loading. The reduced glucose utilization was reversed by manganese supplementation. Manganese administration to diabetic subjects has a hypoglycemic effect (Belyaev, 1938; Rubenstein, 1962) and both pancreatectomy and diabetes have been correlated with decreased concentrations in blood and tissues (Babenko and Karplyuk, 1963; Kosenko, 1964).

MOLYBDENUM

Deficiency

Although molybdenum deficiency has never been reported in man, molybdenum has been shown to be essential for the diet of lambs, chicks and turkey poults (Ellis et al., 1958; Higgins et al., 1959; Reid et al., 1956). In 1953 (deRenzo et al., 1953a; deRenzo et al, 1953b, Richert and Westerfield, 1953) it was shown that the

flavoprotein enzyme xanthine oxidase is a molybdenum-containing
metalloenzyme which requires molybdenum for its activity

Dental Caries

    Claims have been made based on epidemiological studies carried
out in Hungary (Adler and Straub, 1953) and New Zealand (Ludwig et
al., 1960; Healy et al., 1961) that molybdenum decreases the in-
cidence and severity of dental caries and that it might enhance
the well-established effect of fluoride. In two of the earliest
experiments with rats, a reduction in caries was reported when
molybdenum was supplied during tooth formation. Other experiments
failed to show an effect when rats fed a cariogenic diet were given
supplemental molybdenum at levels up to 50 ppm (Westerfield and
Richert, 1953; Buttner, 1961; Buttner, 1963; Malthus et al., 1964).

NICKEL

    Concentrations of serum nickel become increased in patients
following acute myocardial infarction, stroke, burn and septicemia
(Sunderman et al., 1970; McNeely et al., 1971; Sunderman et al.,
1972). Diminished average concentrations of serum nickel are found
in patients with hepatic cirrhosis and with chronic renal insuf-
ficiency (Sunderman et al., 1970; McNeely et al., 1971). Signifi-
cant concentrations of nickel are present in DNA (Eichhorn, 1962;
Wacker et al., 1959) and RNA (Bertrand and Macheboeuf, 1926; Al-
lison and Lancaster, 1964; Wacker et al., 1963) from phylogeneti-
cally diverse sources. Nickel and other metals which are present
may help stabilize the structure of nucleic acids.

SILICON

Aging

    Leslie et al. (1962) have found a decrease in the silicon
content of rat skin with age. Similarly, in human skin the silicon
content of the dermis diminishes with age (Brown, 1927; MacCardle
et al., 1943). The silicon content of the aorta decreases with
age (Jones and Handreck, 1967), and arterial wall silicon decreases
with the development of atherosclerosis (Loeper et al., 1966).
Schwarz (1974) has suggested that silicon may have a role in muco-
polysaccharide metabolism. Silicon is a constituent of certain
glycosaminoglycans and polyuronides where it is apparently bound
to the polysaccharide matrix (Schwarz, 1974). Silicon may link
portions of either the same polysaccharides to each other, acid
mucopolysaccharides to each other, or acid mucopolysaccharides to
proteins. Silicon may function as a biological cross-linking agent
and may contribute to the structure and resilience of connective
tissue.

SELENIUM

Cancer

Sodium selenide, as well as the antioxidants vitamin E and
ascorbic acid, prevented skin tumor formation in mice (Shamberger,
1966; Shamberger, 1970). Sodium selenite-supplemented torula yeast
diets also significantly decreased mouse skin tumor formation in-
duced by 7,12-dimethylbenzanthracene-croton oil (Shamberger, 1970).
In addition, there is an inverse relationship between the forage
crop content of selenium and human cancer mortality in several
states (Shamberger and Willis, 1971; Shamberger et al., 1974a;
Shamberger et al., 1976). When the mortality from several types
of cancer are compared in the high- and low-selenium areas, there
are much lower death rates from the types of cancer in organs which
might come into contact with selenium than in the organs which do
not come into contact directly with selenium. Digestive organ
cancer death rates are much lower in the high selenium areas
(Shamberger and Willis, 1971; Shamberger et al., 1974a; Shamberger
et al., 1976). Similar results have been reported by Schrauzer
et al. (1977) when he found an inverse relationship between sele-
nium in blood bank blood and human cancer mortality.

Selenium as well as other antioxidants may be stabilizing or
breaking down malonaldehyde, a product of the peroxidative break-
down of unsaturated fatty acids. Malonaldehyde is a carcinogen
which is present in large amounts in certain types of meats, but
not in fruits or vegetables (Shamberger et al., 1974b; Shamberger
et al., 1977a). This finding may have some relationship to the
elevated colon cancer death rates in countries with high beef con-
sumption. Malonaldehyde has also been found to be mutagenic in
the Ames system (Mukai and Goldstein, 1976; Shamberger et al.,
1979a). The mutagenicity of malonaldehyde has been reduced by the
antioxidants selenium, vitamin E, BHT and ascorbic acid (Shamber-
ger et al., 1979a).

Selenium in the form of sodium selenite, as well as other
antioxidants, reduced carcinogen-induced chromosome breakage when
compared to the controls (Shamberger et al., 1973a). Lower blood
selenium levels have been observed in patients with gastrointestinal
cancer or liver metastases (Shamberger et al., 1973b). McConnell
et al. (1975) have reported lower blood selenium concentrations in
almost all types of cancer patients.

Several reductions in tumor incidence induced by different
carcinogens have been observed with selenium supplemented diets:
Clayton and Baumann (1949) found that the inclusion of 5 ppm of
selenium in a purified diet reduced the incidence of liver tumors
in rats induced by N^1-methyl-p-dimethyl-aminoazobenzene. Marshall
et al. (1978) have reported similar results. Dietary selenium

has also reduced N-2-fluorenyl-acetamide-induced cancer in vitamin-E
supplemented rats (Harr et al., 1972). Griffin and Jacobs (1977)
have reported a decrease of liver tumors induced by 3^1-methyl-4-
dimethylaminoazobezene. Schrauzer and Ishmael (1974) observed a
lower incidence in spontaneous mammary tumors in C_3H/St mice
supplemented with 2 ppm of selenium in the drinking water for 15
months. Jacobs et al. (1977) have observed a marked reduction in
the number of colon tumors per animal and in the total number of
tumors induced by 1,2-dimethylhydrazine when 4 ppm of selenium
as sodium selenite was added to the drinking water.

Heart Disease - Animal Studies

    Rats (Godwin, 1965) and lambs (Godwin and Fraser, 1966) fed
Se deficient diets develop abnormal electrocardiograms accompanied
by blood pressure changes. In lambs, progressive development
of a characteristic electrocardiogram abnormality is seen, after
the animals had been on the deficient diet for a few weeks. Just
before death the ECG pattern became grossly abnormal in some cases,
a rise in the T-wave giving way to an elevated S-T segment, similar
to that seen frequently in myocardial infarction in man.

    In piglets born of Se and vitamin E deprived sows, progressive
vascular changes were observed. Connective tissue lesions of the
heart were noted which characterize mulberry heart disease of swine
and may result in sudden death of the animal. The disease has been
termed "mulberry heart disease" because the affected animals fre-
quently have extensive cardiac hemorrhage that gives the organ a
reddish-purple gross appearance comparable to that of a mulberry.

    Cadmium has been observed to cause hypertension in rats (Perry,
1973). The Cd pressor effect caused by 2.5 and 10 ppm of cadmium
in the drinking water can be reversed by 3 ppm of selenium in the
drinking water (Perry, 1974).

Heart Disease - Human Epidemiology

    Kubota et al. (1967), after determining the selenium content
of various forage crops, constructed a map of the United States
showing the generalized regional distribution pattern of Se in
forage crops. The male and female 54-64 age specific death rates
per 100,000 for cardiovascular-renal, cerebrovascular, coronary,
and hypertensive heart disease were all significantly lower
(P<0.001) in the high selenium area than the low Se area (Shamberger,
1975). For the most part there was an inverse gradation in mortal-
ity and Se bioavailability. States with occasionally large amounts
of selenium in the drinking water such as Colorado, North and South
Dakota and Utah had the lowest hypertensive death rate. Although
selenium in the drinking water could be an important factor in
these states with the lowest white male hypertensive death rate,

certainly this observation may be unrelated to selenium in the drinking water and may be due to as yet unrecognized environmental factors.

Comparisons among 9 types of heart mortality in 17 cities showed significantly lower death rates due to arteriosclerotic and hypertensive heart disease for both males and females (Shamberger et al., 1975). Non-white mortality rates were markedly greater for cerebrovascular and hypertensive heart disease. The non-white are located in the inner city areas where there is a greater chance of airborne cadmium pollution, which has been previously correlated with arteriosclerotic and hypertensive heart disease.

In another epidemiological study (Shamberger et al., 1978) selenium, cadmium, zinc, copper, chromium, arsenic and manganese estimated intakes in 25 countries were related to the 1973 age-specific mortality from hypertensive heart disease, cerebrovascular heart disease and diseases of the arteries and capillaries in those 25 countries. In general, a negative significant correlation co-efficient was seen with Se and ischemic heart disease and a posi-tive correlation was observed with Cd and ischemic heart disease. Both of these elements compete for the same sulfydryl binding site and could be interrelated. Some other metals, chromium and manganese, are also related to heart disease mortality. Japan and Hong Kong, which were not included in the study with the 25 coun-tries because of racial differences, had a very low Cd/Se ratio and a low incidence of ischemic heart disease. In autopsy speci-mens, Shamberger et al. (1977b) found no difference in the Cd/Se ratio of the kidneys from those who died from heart disease and those who died from other causes. Voors et al. (1978) have done a similar study on autopsy kidneys but related heart weight as an index of hypertension of the quotient of the tissue concentrations of Cd/Se Zn. A positive correlation was observed (p<0.01, by a one-tailed test).

In another epidemiological experiment Shamberger et al. (1979b) observed that male and female 55-64 age-specific death rates per 100,000 for cardiovascular-renal, coronary and hypertensive heart disease had significant inverse relationships to the mean blood bank selenium concentration in 19 states. In addition, a positive Chi square association was observed between blood bank selenium and forage crop selenium. Bjorksten (1979) has recently reported that the countries in Finland having more selenium in the drinking water had only about 1/7 of the heart disease mortality than the countries with low selenium water.

Clinical Trials

Patients taking selenium-tocopherol capsules (Tolsem) have shown reduced incidence of anginal pain in 22 of 24 cases

(Villalon, 1974). Keshan disease was first discovered in China
in 1935. This is a type of congestive heart failure with the most
susceptible people being children below 15 years of age, women of
child-bearing age in the northern privinces, and children of 2-7
years of age in the southern affected areas. Encouraging results
were obtained in trials using sodium selenite to prevent the
disease by researchers at Sian Medical College in 1965. This led
their researchers to conduct a four-year clinical trial conducted
by the Keshan Disease Research Group of the Chinese Academy of
Medical Sciences (1979). Children received sodium selenite tab-
lets or placebo once a week, the dosage being: 1-5 year olds,
0.5 mg and 6-9 year olds, 1.0 mg. Of the 36,603 Se treated chil-
dren, 21 cases occurred during the four years of investigation.
Of these only 3 of them died and 1 became chronic up to the end
of 1977, whereas of the 9,642 children in the control group, 107
cases occurred of which 53 were fatal and 6 still had insufficient
heart function. These results indicate that administration of
sodium selenite not only decreases morbidity but also alleviates
clinical symptoms and improves the prognosis.

TIN

     Although the biological function of tin is unknown, tin has
a number of chemical properties that offer possibilities for a
biological function. Tetravalent tin has a strong tendency to form
coordination complexes with four, five, six and possibly eight
ligands. Tin (Schwarz et al., 1970) may contribute to the tertiary
structure of proteins or other components of biological importance.
Tin could participate in oxidation-reduction reactions in biologi-
cal systems because the $Sn^{2+} \rightleftharpoons Sn^{4+}$ potential of 0.13 volts is
within the physiological range near the oxidation-reduction po-
tential of flavine enzymes.

VANADIUM

Lipid and Carbohydrate Metabolism

     Vanadium reduced the in vivo cholesterol concentration in
plasma from rabbits and from human plasma (Curran et al., 1959;
Mountain et al., 1956; Lewis, 1959; Curran and Costello, 1956;
Korkor, 1965). Decreased plasma phospholipids were also observed.
Workmen exposed to an industrial source of vanadium had slightly
but significantly lower (205 mg/100 ml) concentrations of phospho-
lipids than that of controls (228 mg/100 ml). In older human pa-
tients and in patients with hypercholesterolemia or ischemic heart
disease, vanadium does not lower serum cholesterol (Schroeder et
al., 1963; Curran and Burch, 1967; Dimond et al., 1963; Somerville
and Davies, 1962; Azaroff and Curran, 1967). In younger subjects
vanadium interferes with cholesterol synthesis by inhibiting utili-
zation of mevalonic acid (Curran et al., 1959) and also accelerates

cholesterol catabolism. Vanadium also inhibits the synthesis of cholesterol from acetate but not from squalene (Azaroff and Curran, (1967), because vanadium inhibits squalene synthetase catalyzing the conversion of labeled farnesyl pyrophosphate to squalene (Azaroff et al., 1961). Plasma triglyceride concentrations are significantly increased in vanadium-deficient chicks (Hopkins and Mohr, 1974; Hopkins, 1974). Glucose is more rapidly turned over after vanadium administration (Meeks et al., 1971). As glucose is a precursor of triglyceride, this may explain why triglyceride concentrations are lower.

Dental Caries

Microradioautography indicates that $^{48}V_2O_5$ injected subcutaneously into mice is highly concentrated in the areas of rapid mineralization in tooth dentin and bone (Soremark and Ullberg, 1962). After injection, vanadium is also incorporated into the tooth structure of rats and retained in the molars up to 90 days (Thomassen and Leicester, 1964). Rygh (1949a; 1949b; 1949c) has reported that the addition of vanadium and of strontium to specially purified diets promoted mineralization of the bones and teeth and reduced the number of carious teeth in rats and guinea pigs. Vanadium protected against caries in hamsters fed a cariogenic diet when vanadium was administered as V_2O_5, either orally or parenterally (Geyer, 1953). Intraperitoneal administration of vanadium to rats during tooth development was effective in reducing dental caries (Kruger, 1958). However, in three other rat studies, administration of varying levels of vanadium in the drinking water was not successful in decreasing caries incidence (Buttner, 1963; Muhlemann and Konig, 1964; Muhler, 1957; Tank and Storrick, 1960). Ten ppm of vanadium actually increased caries when young hamsters were given a cariogenic diet (Hein and Wisotzky, 1955).

ZINC

Acrodermatitis Enteropathica

Acrodermatitis enteropathica (AE) is a serious illness which starts in early infancy after weaning. The disease becomes gradually more insidious and is recognized by changes in skin and hair. There are also gastrointestinal symptoms, especially diarrhea. Skin lesions are localized on the hands and feet, as well as near the orifices, especially in the perioral and in the diaper regions. During inactive phases the skin in these areas is dry, erythematous, peeling, sometimes psoriasis-like, while during the active periods, it is vesicopustular, red and erosive. The first treatment of this disease was with chlorquinaldol, but side effects such as optic atrophy have been observed. Moynahan (1974) has reported excellent results with oral zinc with several AE patients. Michaelsson (1974) has reported similar results. Oral zinc in combination with

vitamin A has proven to be more effective in treating acne than
vitamin A alone (Michaelsson et al., 1974).

The AE lesion in humans may be related to some of the known
zinc deficiency symptoms in animals. Gross epithelial lesions and
alopecia develop in rats and mice with zinc deficiency. Histolo-
gical studies revealed a condition of parakeratosis, i.e., a hyper-
keratinization of the epithelial cells of the skin (Follis et al.,
1941). Rats with more severe zinc deficiency have scaling and
cracking of the paws with deep fissures, coarseness, and loss of
hair, and dermatitis (Orten, 1966). Parakeratosis occurs around
the eyes and mouth and on the scrotum and lower parts of the legs
(Miller et al., 1965). Zinc-deficient ruminants show similar
patterns (Underwood and Somers, 1967; Blackmon et al., 1967). Zinc
deficiency is evident in sheep through changes in the wool growth,
hoof structure and horns (Mills et al., 1967; Underwood and Somers,
1969). The wool fibers lose their crimp and become thin and loose
and readily shed.

In the Zn-deficient chick (O-Dell and Savage, 1957; O'Dell
et al., 1958), turkey poult (Kratzer et al., 1-58), and pheasant
(Scott et al., 1959), feathering is poor and abnormal and dermatitis
is present. The epidermal changes seen in zinc-deficient animals
may be related to vitamin A deficiency. Zinc is necessary to main-
tain a normal concentration of rat plasma vitamin A (Smith et al.,
1973). Zinc is known to be necessary for normal mobilization of
vitamin A from the liver.

Reproduction

Hypogonadism, with suppression in the development of the
secondary sexual characteristics, is conspicuous of zinc deficiency
of young men in parts of Iran and Egypt (Prasad et al., 1961;
Prasad et al., 1963). Hypogonadism also occurs in Zn-deficient
bull calves (Miller et al., 1966; Pitts et al., 1966), kids (Miller
et al., 1964) and ram lambs (Underwood and Somers, 1969). Human
spermatozoa have high zinc levels. Zinc-deficient male rats (Follis
et al., 1941) have been observed to have atrophic seminiferous
tubules. Zinc deficiency also retarded development of the testes,
epididymus, prostate and pituitary glands with atrophy of the tes-
ticular germinal epithelium. High concentrations of zinc are
necessary for the final stages of sperm maturation and seem to be
essential for the maintenance of spermatogenesis and for the sur-
vival of germinal epithelium (Underwood and Somers, 1969; Millar
et al., 1958, and Wetterdal, 1958). In female rats, zinc defi-
ciency caused a severe disruption of estrus (Hurley and Swenerton,
1966). Zinc deficiency during gestation of rats greatly reduces
the number of young that are born alive (Apgar, 1968a; Apgar,
1968b).

Hypogeusia

     Several adult patients with low serum zinc developed decreased
taste acuity (hypogeusia). Oral zinc supplementation relieved
this program (Schechter et al., 1972; Cohen et al., 1973).

Atherosclerosis

     Twelve of thirteen patients with advanced vascular disease
who were given zinc sulfate orally up to 29 months, showed clinical
improvement; nine returned to normal activity. In a similar study
by Henzel et al. (1968), about half of the patients improved. Plas-
ma and hair zinc concentrations are usually significantly subnormal
in patients with atherosclerosis and myocardial infarction (Pories
et al., 1967; Volkov, 1963; Wacker et al., 1956). Atherosclerosis
may be an expression of inadequate arterial repair (Pories et al.,
1967), which like wound healing in skin, muscle, and bone is re-
lated to zinc concentrations.

     Within a day of onset of acute myocardial infarction serum
zinc concentrations fall sharply and then rise to normal values
within 7 to 10 days (Handjani et al., 1974).

Wound Healing

     Rats fed zinc accidently have an accelerated rate of wound
healing in rats (Pories et al., 1968; Pories and Strain, 1966).
Seriously burned patients have subnormal hair zinc concentrations
(Pories and Strain, 1966). Wound healing is impaired in Zn-
deficient calves (Miller et al., 1965) and rats (Sandstead and
Shepard, 1968). Although the mode of action of zinc in tissue
repair is unknown, zinc is preferentially concentrated in healing
tissues in rats, both in skin and muscle wounds (Savlov et al.,
1962), and in bone fractures (Calhoun and Smith, 1968). This con-
centration suggests a heightened zinc requirement for tissue syn-
thesis during the healing process. Wound healing showed a decreased
rate in zinc-deficient animals (Rahmat et al., 1974). Some investi-
gators have reported no effect of supplemental zinc on wound healing
in rats (Murray and Rosenthal, 1968; Quarantillo, 1971).

Cancer

     In patients with malignant tumors subnormal plasma zinc con-
centrations have been reported (Addink, 1960; Addink and Frank,
1959; Vidbladh, 1950). Leukocyte zinc concentrations are markedly
reduced in patients with chronic leukemia. Zinc concentrations
are raised to normal by zinc injections and rise to normal during
clinical remission (Gibon et al., 1954). The zinc content of the
leukocytes also decreases in patients with a variety of neoplastic
diseases (Szmigielski and Litwin, 1964).

The inhibition of chemical carcinogenesis in the hamster cheek pouch by dietary zinc deficiency has been reported (Poswillo and Cohen, 1971). DeWys et al. (1970) reported the inhibition of Walker 256 carcinosarcoma by dietary zinc deficiency. McQuilty et al. (1970)have reported similar results. Because zinc is necessary for growth of normal tissues, zinc may also be necessary for tumor growth. The growth of P 388 leukemia in weanling mice was depressed after the third post-transplantation day (Barr and Harris, 1973). Growth of a transplantable hepatoma induced by 3^1-methyl-4-dimethylaminoazobenzene was significantly reduced in rats maintained on a low-zinc diet (Duncan et al., 1974).

Growth

Retardation of growth in young animals was observed in the original demonstration of zinc deficiency in rats (Todd et al., 1934) and has been a conspicuous feature of this deficiency in all subsequent studies. The growth inhibition results partly from reduced food intake and partly from an impaired utilization of the food that is consumed and absorbed. Male dwarfs living in restricted areas in the Middle East are the only evidence of zinc deficiency in man of sufficient severity to produce deficiency symptoms (Prasad et al., 1961).

Sickle Cell Anemia

Zinc plasma concentrations were compared in patients with sickle cell anemia in a steady state, in sickle cell crisis and in controls (Niell et al., 1979). Lowest zinc plasma levels were observed during sickle cell crisis. Low values of plasma zinc were also seen when patients had their sickle cell anemia in a steady state. The drop in plasma zinc seen in sickle cell patients could be related to stress.

NEED FOR FURTHER RESEARCH

Although several trace elements have tentatively been related to health and disease effects in animals and humans, much additional research is needed to firmly establish these relationships. To accomplish this goal one of the greatest needs is to develop analytical methods which will detect trace elements at concentrations which occur in biological materials. When these methods are developed rapid progress in trace element research could be made.

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ROLE OF SPECIFIC NUTRITIONAL COMPONENTS

ON PLASMA LIPIDS, LIPOPROTEINS AND CORONARY HEART DISEASE

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INTRODUCTION

Heart and blood vessel diseases are the major causes of
death in this country. They accounted for over 50 percent of all
deaths annually; nearly three times the death rate from cancer,
the next highest cause. Cardiovascular diseases cause over 60%
of all deaths among people over 65 years of age and over 150,000
deaths per year in individuals below age 65. An estimated 30
million persons in the United States have diseases of the heart
and blood vessels; this results in a huge burden of acute and
chronic illness and disability. Some 27 million of these victims
of cardiovascular disorders suffer from hypertension, 4 million
from coronary heart disease, and 1.8 million from rheumatic heart
disease. Approximately eight out of every one thousand children
are born with congenital heart disease and half of these do not
survive past one year of age.

However, during the last 25 years there has been over a 30%
decrease (Figure 1) in cardiovascular death rate (age adjusted).

The number of deaths per year from major cardiovascular
disease dropped below one million for the first time since 1967
in 1977, although the population in the United States has in-
creased and is comprised of a larger proportion of senior citizens.
The reasons for this are not clear, but are more than likely due
to a combination of factors: (1) improved and earlier detection,
treatment and rehabilitation of patients with cardiovascular
disease and its related risk factors, (2) better emergency care
of victims of myocardial infarction attacks, (3) a larger segment
of the mass population being more aware and concerned about

Year	Rate per 100,000 population	
	CVD	NonCVD
1950	425.6	415.9
1975	299.2	343.1
Percent Change	-29.7	-17.5

*PROJECTED
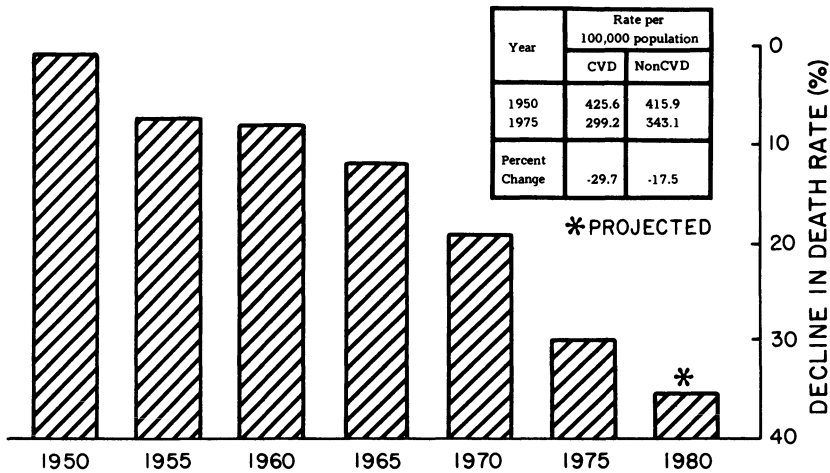
Fig. 1. Cardiovascular death rate (age adjusted over a 20 year
         period (adapted from the Fourth Report of the Director,
         NHLBI, 1977).

physical fitness, health, nutrition and diets and (4) more effec-
tive education of the mass population about controlling primary
and secondary risk factors associated with coronary heart disease
(CHD). Despite such progress, major cardiovascular disease still
leads all other causes of death in the United States, with more
than a half a million persons having been predicted to die of
coronary heart disease in 1980.

     This chapter will discuss the role of specific dietary
components on plasma lipid and lipoprotein concentrations. The
major plasma lipids of interest are total cholesterol (free
cholesterol + ester cholesterol) and the triglycerides. Rarely
is measurement of plasma phospholipids helpful, except for in-
stances of obstructive liver disease. When one or more of these
major classes of plasma lipids is elevated, a condition exists
that is referred to as hyperlipidemia. A problem arises in
defining what levels of plasma lipids separate persons with ele-
vated blood fats from the rest of the "normal" population. Cut-
off valves for plasma lipids are assured to represent the 95TH
percentile of their distribution in a given population. Thus,
before one can address the question of what is adequate dietary or
drug therapy for the control of serum lipid and lipoprotein levels,
one needs to first consider the degree to which blood lipids
should be lowered. In other words, at what level should the
clinician consider a sample "hyperlipidemic?" Many clinical
laboratories and practicing physicians still use normal ranges in
evaluating whether or not a sample can be considered normal or
abnormal. These normograms are based on sampling of "apparently

normal" individuals and arbitrarily defining hyperlipidemia as being present when the plasma cholesterol and/or triglycerides are above the 95^{TH} percentile value for the population to which the persons belong. Unfortunately, because of the way we have defined "normal" in the past, many abnormal test results do not correlate very well with disease states or health-risk conditions on an individual basis. Thus, the "normal" range may not be a "healthy" level. While a cholesterol value of 250-280 mg/dl may be within the 95^{TH} percentile of the distribution of an apparently normal male population between the ages of 51-59 in the U. S., about 40-50% of these individuals eventually develop CHD. One of the most frequent and perhaps most frustrating questions confronting the clinician regarding laboratory tests is "What is normal?" In recent years the term "normal range" has been replaced by "reference range." "Upper limit of normal" is being replaced by "referent value," "critical value" or "cut-off point." There are at least five major considerations that the reference values depend upon: (1) the reference population and the way that it was chosen, (2) the environmental and physiological conditions under which the specimens were obtained, (3) the techniques and timing of specimen collection, transportation, preparation and storage, (4) the analytical method that was used, with data regarding its accuracy, precision, and quality control, and (5) the data set that was observed and the reference intervals that were derived (Galen, 1977). There also is the consideration for sub-selection based on ethnic background, geographical location, occupation, body weight, sex, age and other factors such as smoking, alcohol intake and dietary habits. The reference intervals, 2.5 to 97.5^{TH} percentiles, can be compared to the Gaussian estimation of 95% of the population, which is included within $\pm$ 2 S.D. from the mean. The 2.5 and 97.5^{TH} and 0.5 and 99.5^{TH} percentiles are frequently used to define the laboratory reference intervals for a chemistry test. The clinician dealing with a single patient, however, does not really care about standard deviations or percentiles. He is most interested in knowing whether the test result predicts a particular disease state or implies risk of morbidity or mortality. It is here that the predictive value approach may prove to be of utmost significance. Referent values for serum lipids and lipoproteins should be established that are highly predictive of disease or disease risk, irrespective of the "normal distribution."

There are four components that are important in determining the accuracy of a laboratory diagnostic test: sensitivity, specificity, predictive value, and efficiency. Sensitivity indicates the frequency of positive test results in patients with a particular disease, whereas specificity indicates the frequency of negative test results in patients without that specific disease. The predictive value of a positive test result indicates the frequency of diseased patients in all patients with positive test results. The

predictive value of a negative test result indicates the frequency
of nondiseased patients in all patients with negative test results.
The efficiency of a test indicates the percent of patients cor-
rectly classified by the test. In screening for disease, we are
most interested in predictive value of the positive result. In
CHD, this means establishing predictive values for total choles-
terol, triglycerides, LDL, LDL-cholesterol, HDL, HDL-cholesterol,
etc. Epidemiological studies (Kannel et al., 1971; Glueck et al.,
1976) show that CHD risk is linear down to a cholesterol concen-
tration of 180 mg/dl. While this philosophical approach to patient
care is pragmatic, it is an idealistic approach to use blood
lipids as "predictors" or diagnostic aide of CHD on an individual
basis. This is because coronary atherosclerosis is a complex
disease which is multifaceted in origin. Although intensive
research efforts have begun to elucidate various facets of the
etiology, development and prognosis of the pathogenesis of athero-
sclerosis, we are still not sure how the initiating and accelera-
ting factors associated with this degenerative disease process
interact and manifest themselves. One reason is that the disease
process itself is complex, probably developing through a multitude
of processes. Large scale epidemiological studies from this
country and others have clearly shown that certain predisposing
factors are associated with CHD. These factors may not be causes
of CHD, yet because of their close association, demonstrated by
statistical means with cardiovascular disease, are called "risk
factors," which have been categorized as primary and secondary.
(Table I)

Table 1. Coronary Heart Disease Risk Factors

Primary	Secondary
1. Genetic predisposition for CHD	1. Overweight
2. High blood lipids	2. No exercise
3. Hypertension	3. Chronic stress
4. Smoking	4. Imbalanced diet
	5. Diabetes mellitus
	6. Hypothyroidism
	7. Renal disease
	8. Birth control pill

One can further define the risk factors into high, above
average and normal risk as shown in Table 2.

Table 2. Degree of CHD Risk Based on Risk Factors

Risk Factors	Risk for CHD		
	Normal	Above Average	High
Total chol. (mg/dl)	<220	220-240	>240
Blood Press. (mm Hg)			
Diastolic	80	90	105
Systolic	120	140	160
Smoking (pks/day)	0	1	2
Fam. History for CHD	Normal	Mod. high	High
Body weight	Normal	10% above normal	20% above normal
Exercise	Regularly	Infreqently	Never
Chronic stress	Minimal	Mod. exposure	High exposure
Diet	Low chol.	Mod. chol.	High chol.
	Low sat. Fat	Mod. sat. fat	High sat. fat
	High fiber	Mod. fiber	Low fiber

     While this concept for categorizing risk for CHD into high,
above average and normal risk is convenient, it is a simplistic
approach to assessing a patient's risk for CHD. For example, it
is well established that elevated serum cholesterol concentration
is associated with increased risk of atherogenesis and CHD (Nichols
et al., 1977, Glueck and Connor, 1977; Davis and Havlik, 1977
and Grundy, 1977). Persons with disorders characterized by
elevated levels of serum cholesterol or low density lipoprotein
(LDL) have a strikingly high incidence of CHD, which often is
evident at a young age (Stone and Levy, 1972). If the familial
β-hyperlipoproteinemic condition is of a homozygote origin, fatal
complications of premature atherogenesis occur before age 30

(Fredrickson et al., 1967; Fredrickson and Levy, 1972). From epidemiological studies and basic and clinical research, there appears to be little doubt that hypercholesterolemia exists as an independent risk factor for coronary artery disease (CAD) (Medalie et al., 1973; Wilhelmsen et al., 1973; Gordon et al., 1974; Johnson et al., 1968). Data from Mainland China show that the mean serum cholesterol level in normal people is 136 mg/dl, while in CHD patients it is 190 mg/dl (Van Die Redaskie, 1973). Thus, in a country with a low rate of CHD, there also appears to be a positive relationship between CHD and cholesterol levels. The relationship is continuous and, in fact, is exponential as the level of serum cholesterol increases. There are no cut-off points to speak of. Therefore, in assessing a person's risk for CHD using risk factors, they should be considered as a continuum.

Another concept that should be remembered when using CHD risk factors is that statistically valid data obtained on large popu-lations and in prospective epidemiological studies are not neces-sarily directly applicable to an individual. As discussed by Blackburn (1974), there is a large variation among individuals in these predisposing factors and within a single individual during his life span. Factors acting throughout the entire period of the developing degenerative disease are difficult, if not impossible, to evaluate. At any one time, primary risk factors (hypertension, smoking, elevated blood cholesterol level, family history for coronary artery disease) and secondary risk factors are simultan-eously involved in the pathogenesis.

Still another difficulty is that differences in food habits have not been adequately demonstrated within the United States between people with and without coronary disease, although habitual diet is related to serum cholesterol concentrations in different populations. The Framingham Study seems to suggest that individual genetic, personal and environmental differences may override the small differences in dietary intake within any one culture (Kannel et al., 1971).

In spite of these possible overriding factors, there can be little doubt that the initiation, development, progression, and regression of atherosclerosis are closely associated with nutri-tional factors. The evidence accumulated over the last 40 years in animal, clinical and epidemiological studies is impressive and persuasive. Nutrition has an important influence on several of the risk factors, particularly serum lipids, lipoproteins, overweight, hypertension, endocrine imbalance and other metabolic conditions closely associated with atherosclerosis. With appropriate nutri-tional adjustments, these conditions associated with degenerative vascular disease can be altered and controlled. Whether the modi-fication of these risk factors by diet will ultimately lead to

lower mortality and morbidity from CHD is yet to be proven. Four
primary prevention studies (Los Angeles Veterans Administration
Study, Finnish Diet Study, Oslo Diet-Heart Study and U. S. Diet-
Heart Study) using dietary measures have shown a trend toward
favorable results (Dayton et al., 1969; Miettinen et al., 1972;
Laren, 1966; National Diet-Heart Study Final Report, 1968). Re-
cently, Frantz et al., 1975 from the Minnesota Coronary Survey
reported that in the cholesterol-lowering dietary trial, men 50
years of age showed less mortality and morbidity from CHD events
than the control group.

It is now believed that dietary treatment in this country
should be initiated when the serum cholesterol level is >220 mg/dl
in adults and above 200 mg/dl in children (Food and Nutrition
Board, 1972; Connor et al., 1973). The upper limit of serum tri-
glyceride levels is less clear. Perhaps levels in excess of 130-
150 mg/dl can be considered unhealthy (Rhoads et al., 1976). The
rationale for initiating dietary therapy when the above limits are
exceeded is to prevent or minimize the development of CHD, since
atherosclerosis seldom occurs with total cholesterol concentration
<200 mg/dl over the life span of an individual (Connor and Connor,
1972), unless other risk factors such as genetics, high blood
pressure, smoking and obesity are playing a dominant role.

While serum lipids are not to be neglected, the influence of
other risk factors associated with CHD should also be simultan-
eously considered. They include smoking, hypertension, obesity,
stress, lack of physical activity, hormonal imbalances and other
primary disease states directly or indirectly associated with
abnormal lipid and lipoprotein metabolism.

From a physiological viewpoint, it is difficult to talk about
lipids as a separate entity from lipoproteins, since all lipids are
associated with protein components to form soluble complexes in
aqueous milieu (see Table 3). While it is easy and convenient to
consider only the serum lipid levels when monitoring the effect of
a prescribed diet, attention should be focused on the serum lipo-
proteins for at least two reasons:

    a)   Lipids are transported as lipoproteins and should
           be considered as lipid abnormality that is classi-
           fiable as different types of dyslipoproteinemic
           conditions.

    b)   LDL-cholesterol concentration is positively related
           to the risk of CHD while HDL-cholesterol concentra-
           tion is negatively related to the risk of CHD. Thus,
           the progress of an individual on the diet should be
           assessed by not only the serum total cholesterol con-
           centrations but by the LDL or LDL-cholesterol and HDL

Table 3. Physical, Chemical and Physiologic Description of Plasma Lipoproteins[a]

	Chylomicron	Very-Low-Density Lipoproteins	Intermediate Density Lipoproteins	Low-Density Lipoproteins	High Density Lipoproteins
Electrophoretic Nomenclature	Chylomicron	Pre-β-Lipoprotein	Midband Lipoprotein	β-Lipoprotein	α-Lipoprotein
Density (g/ml)	0.95	0.95-1.006	1.006-1.019	1.019-1.063	HDL_2=1.063-1.10 HDL_3=1.10-1.25
S_f Rate	>400	20-400	12-20	0-10	---
Electrophoretic Mobility (Paper)	Origin	β-globulin	β-globulin	β-globulin	$α_1$-globulin
Molecular Diameter (A°)	300-5000	~400	~245	200	70-100
Major Lipid Transported	Exogenous Triglycerides	Endogenous Triglycerides	Cholesterol	Cholesterol	Phospholipids
Major Apoproteins	Apo B,C-I,II, III	Apo B,C-I,II, III, Arginine-rich	Apo B	Apo B	Apo A-I,II
Minor Apoproteins	Apo A-I,II	Thin-Line Protein Apo A-I,II			Apo C-I,II,III Thin-Line Protein Arginine-Rich

From Jackson et al. (1976), Osborne and Brewer (1977) and Minamisono et al. (1978)

or HDL-cholesterol levels. When hyperlipidemia is
defined in terms of the class or classes of plasma
lipoproteins that are considered to be elevated, then
the term hyperlipoproteinemia is applied. The occur-
rence of hyperlipoproteinemia may be primary (gene-
tically determined) or may be secondary to various
metabolic dysfunctions, such as hypothyroidism, dia-
betes mellitus, nephrosis, renal failure, obstructive
liver disease, idiopathic hypercalcemia, acute pancrea-
titis, gout, alcoholism, or dysglobulinemia. If an
underlying cause is identified, then the condition is
said to be secondary; otherwise, it is called primary
hyperlipoproteinemia. (Table 4)

Elevations of plasma cholesterol, triglyceride, and lipoproteins
are amenable to dietary control. Diet is fundamental to the treat-
ment of all forms of primary hyperlipoproteinemia. Even when the
physician deems it necessary to use a drug, the diet must be con-
tinued since the effect of the diet and medication are approxi-
mately additive. In many instances, elevations of plasma choles-
terol or triglyceride, or both, can be satisfactorily controlled by
diet.

Generally, when lowering one serum lipid (i.e. either choles-
terol or triglyceride), the other decreases also. Concomitantly,
the concentration of lipoprotein fractions rich in these materials
(chylomicron, pre-β or β-lipoprotein) drop also. Serum total phospho-
lipid levels usually parallel the changes in serum total choles-
terol concentration; the cholesterol/phospholipid ratios under
normal conditions remain about unity. However, the α-lipoprotein
may not be as predictable. For years the importance of α-lipopro-
teins in the evaluation of dyslipoproteinemias has usually been
neglected, mainly because more emphasis was given to the lipopro-
teins that carried the greatest proportion of triglycerides and
cholesterol, namely chylomicron, pre-β-lipoprotein and β-lipoprotein.
Nikkila (1953) and other investigators (Brunner et al., 1962)
recognized many years ago that MI subjects had lower α-lipoprotein
and higher β-lipoprotein values than did the normal controls.
Recently, numerous reports on population studies indicate that
plasma HDL-cholesterol levels are a major and independent risk
factor for CHD (Rhoads et al., 1976 ; Miller et al, 1977; Castelli
et al., 1977). The point is, it is important to examine the entire
lipoprotein spectrum in evaluating a person's risk to CHD (Corday
and Corday, 1975). A person may have a normal total cholesterol
or β-lipoprotein concentration, but his HDL-cholesterol level may
be inordinately low, thus placing the individual in a high-risk
category for CHD. Whether having a moderately elevated serum
cholesterol concentration (i.e. 275-325 mg/dl) with a high HDL-
cholesterol (i.e. >65 mg/dl) places him in a non-risk category
cannot be answered at this time. Table 5 summarizes factors that

Table 4. Classification of the Types of Primary* Hyperlipoproteinemia[a]

CLINICAL MANIFESTATIONS	TYPE I	TYPE IIa	TYPE IIb	TYPE III	TYPE IV	TYPE V
Incidence	Rare	Common	Common	Uncommon	Common	Uncommon
Lipoprotein Abnormalities by Ultracentrifuge †	Severe chylomicronemia	↑LDL	↑LDL, ↑VLDL	VLDL + LDL of abnormal composition	↑VLDL	VLDL, Chylomicrons
Lipoprotein Abnormalities by Electrophoresis††	Chylomicron band at origin	↑B-Band	B, Pre-B	Broad B (or floating B)	Pre-B	Chylomicrons, Pre-B
Plasma Appearance	Creamy layer over clear plasma	Clear or slightly turbid	Clear or slightly turbid	Turbid	Turbid	Creamy layer over turbid plasma
Cholesterol	↕	↕	↕	↕	↕	↕
Triglyceride	↕	↕	↕	↕	↕	↕
Age of Onset	Infancy and childhood.	At birth, if genetic; early childhood.	At birth, if genetic; early childhood.	Third decade; often after menopause in women.	Third decade or later, can occur in children with obesity or diabetes.	Early adulthood; rare in children.
Familial Forms	Deficiency of lipoprotein lipase.	"Familial Hyperlipoproteinemia" - also called "Familial Hypercholesterolemia", most common hyperlipoproteinemia; expressed in 2 forms: heterozygote (more common disorder, cholesterol concentration elevated, but LDL not grossly abnormal), homozygote (less common but cholesterol and LDL levels markedly exaggerated). Many mild examples are not obviously familial.		Genetic mode uncertain. Often called Broad B disease or remnant disease. B-lipoprotein electrophoretic mobility, but has Pre-B density characteristics. This fraction has a high amount of triglycerides and low amounts of cholesterol.	Second most common form. Often half of adult close relatives will also have Type IV.	When familial, more than half of close relatives have either Type IV or Type V.
Clinical Presentation	Episodes of abdominal pain associated with ingestion of dietary fat, pancreatitis, eruptive xanthomas, hepatosplenomegaly, lipemia retinalis, SLE, lymphoma.	Premature vascular disease, tendon and tuberous xanthomas, arcus cornea in 50% of patients.	Severe forms are like Type IIa, milder Type IIb patterns tend to be accompanied by glucose intolerance, overweight.	Glucose intolerance, tuberoeruptive or planar xanthomas, premature vascular disease; worsened by alcohol.	Abnormal insulin levels, glucose intolerance in about 50%; excess caloric intake common; occasionally eruptive xanthomas, hyperuricemia, hepatosplenomegaly; worsened by alcohol.	Bouts of abdominal pain and pancreatitis, eruptive xanthomas, premature atherosclerotic heart disease, hepatosplenomegaly; excess caloric intake common; hyperuricemia, most patients have glucose intolerance, worsened by alcohol excess.

Table 4. Classification of the Types of Primary* Hyperlipoproteinemia[a]
(Continued)

Rule Out*	Dysgammaglobulinemia, diabetes mellitus, pancreatitis.	Thyroid dysfunction, nephrotic syndrome, obstructive liver disease.	Dysgammaglobulinemia, thyroid dysfunction.	Diabetes mellitus, glycogen storage disease, nephrotic syndrome, pregnancy, Werner's syndrome.	Multiple myeloma, macroglobulinemia, nephrotic syndrome, alcoholism, pancreatitis, gout.
Drug Therapy	No effective drug therapy at present.	May require a combination of diet and drug therapy.			

*PRIMARY (FAMILIAL) FORMS OF HYPERLIPOPROTEINEMIA ARE GENETICALLY DEFINED AS THOSE WHICH RESULT FROM THE GENETIC DEFECT AND ARE NOT THE RESULT OF SEPARATE ENDOCRINE DISORDERS, METABOLIC DISORDERS, IMPROPER NUTRITION, DRUG INTERFERENCE, AND THE LIKE, WHICH ARE SECONDARY (OR ACQUIRED) HYPERLIPOPROTEINEMIAS. ALL SECONDARY FORMS OF HYPERLIPOPROTEINEMIA MUST BE RULED OUT BEFORE CONSIDERING TREATMENT OF PRIMARY HYPERLIPOPROTEINEMIA.

†LDL=Low Density (Beta) Lipoproteins; VLDL=Very Low Density (Pre-Beta) Lipoproteins.

††† = increased concentration. ↓ = decreased concentration. ←→ = no change.

a - From The Cleveland Clinic Foundation Diet Manual: A Key to Nutritional Care (1978)

are known to influence HDL-cholesterol levels.

      Table 5.   Factors Associated With HDL-Cholesterol
              Concentration

Increase HDL-C Levels	Decrease HDL-C Levels
Genetics	Genetics
Weight reduction	Smoking
Physical exercise	High carbohydrate intake
Estrogen-treatment	Overweight
Atromid S(R)	Hypertriglyceridemia
Alcohol	

a
  HDL-C = high-density lipoprotein cholesterol

    There are other reasons for lowering serum lipids besides the
association of high lipid levels with increased incidence in CHD.
Hypertriglyceridemia increases the chances for development of pan-
creatitis, which is a very serious complication of hyperchylomicro-
nemia. Individuals with marked hyperchylomicronemia (Type I hyper-
lipoproteinemia) may develop eruptive xanthomatosis and lipemia
retinalis. With hypercholesterolemia, xanthomas may develop, with
the most common type being tendon xanthomas. Other forms that can
develop are exanthoma tuberosum, planar xanthomas, tuberoeruptive
xanthomas and xanthelasma. Corneal arcus due to lipid deposits in
the corneal stroma may occur.

THE EFFECTS OF SPECIFIC NUTRITIONAL SUBSTANCES ON SERUM LIPID
CONCENTRATIONS

    Before discussing the role of various dietary factors on serum
lipids and atherosclerosis, it must be emphasized that under prac-
tical conditions one cannot examine each dietary factor as a sepa-
rate entity; the effect of each factor on serum lipids is the
result of the total composition and amount of each dietary component
in a meal. Proteins, carbohydrates, fats, minerals and vitamins
all have effects of their own and can have additional effects on
other dietary components, especially on efficiency of intestinal
absorption, transport and metabolism of lipids. Thus, it is impor-
tant to understand the interdependence of nutrients that are mani-

pulated in fat-controlled diets. In this paper, the simplistic
approach will be taken in discussing each nutritional factor as a
separate entity. On occasion, attention will be drawn to the need
for viewing the various nutritional components in relationship to
each other.

There is little question that nutritional factors can affect
serum lipid and lipoprotein levels and patterns. However, it is
difficult to predict accurately the size of these changes, since
the results are influenced by individual genetic differences in
metabolism. Even in animals, such genetic differences occur. In a
recent study (Malinow et al., 1976b) we showed that monkeys on a
basal chow diet supplemented with 0.5% cholesterol for 30 days
showed a wide range of serum cholesterol values (110-1180 mg/dl).
The monkeys were then arbitrarily separated into hypo-, normo- and
hyper-responders, depending upon their cholesterol level; the
dividing concentration being 290 and 990 mg/dl, respectively. It
is clear that hypo- and hyper-responders differ greatly in
their response to dietary cholesterol and that, when they ingest a
diet almost free of cholesterol, their basal cholesterol level is
also lower in the former than in the latter (Figure 2). Similar
findings have been reported in dogs (Mahley et al., 1974), rabbits
(Fillios and Mann, 1956), rats (Imai and Matsumara, 1973), rhesus
(Cox et al., 1958) and squirrel monkeys (Lofland et al., 1972), and
man is no exception.

Secondly, the effect of each individual nutritional component
is influenced by other nutritional components in the diet. It is
important to understand the interdependence of nutrients that are
manipulated in fat-controlled diets. None of these factors (i.e.
total calories, total fats, cholesterol, saturated fat, polyunsatu-
rated fat, proteins, carbohydrates, fiber) can be considered alone.
For example, feeding a high-cholesterol diet with low total fat
will produce different degrees of hypercholesterolemia as compared
to a diet high in cholesterol and high in total fat (Naito and
Gerrity, 1979). Furthermore, the proportion of saturated fat
versus mono- and polyunsaturated fats in the total fat component of
the diet will have differing degrees of influence (Hegsted et
al., 1965).

Hegsted et al. (1965) have shown that serum cholesterol con-
centrations change at various levels of dietary cholesterol with
three different dietary fats (coconut, olive, and safflower oils).
All diets had 38% of the total calories as fat. In each series,
three levels of cholesterol (100, 300 and 700 mg) were given to the
subjects, who were fed a diet containing 38%, 17% and 48% of total
calories as fat, protein and carbohydrates, respectively. Aside
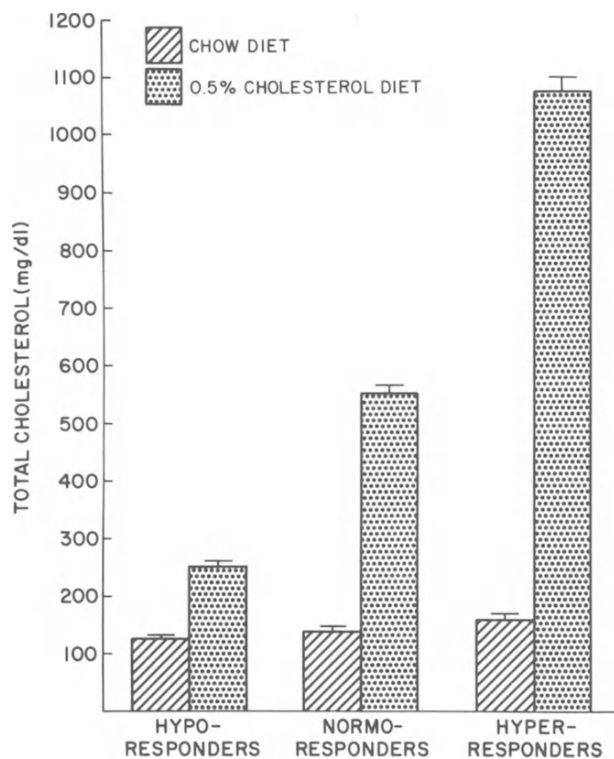
Fig. 2. Non-cholesterol and cholesterol-fed serum cholesterol values of hypo-, normo-, and hyper-responders (Cynomologus monkeys, Macaca fascicularis).

from the fat in lean meat, fish and poultry, all fat came from one
of the three oils. The changes in serum cholesterol levels were
compared to levels established when control subjects were fed a
typical American diet. Regardless of the kind of oil in the diet,
serum cholesterol concentration was reduced about 5 mg/dl for every
100 mg decrease in dietary cholesterol intake. With the olive oil
(26% of calories as monounsaturated fat, with a polyunsaturated/
saturated (P/S) ratio of 0.72%, the cholesterol level was similar
to that of the control subjects. With the coconut oil (32% of
calories as saturated fats, with a P/S ratio of 0.03), serum choles-
terol increased by 40 mg/dl above control levels, while the saf-
flower oil (25% of calories as polyunsaturated fats with a P/S
ratio of 5.00) was 40 mg/dl below the control group at each of the
respective levels of cholesterol intake. Thus, fat intake affects
blood lipids depending on the total dietary fat and the quality of
the fat components in the diet. For example:

1.   Decreasing total dietary fat decreases total plasma
     cholesterol and triglyceride concentrations (Ahrens et
     al., 1957; Keys et al., 1957; Grundy, 1979; Connor and
     Connor, 1977). The degree of this hypolipaemic change
     is highly dependent upon the actual level and type of
     dietary fat consumed, the responsiveness of the indi-
     vidual, the genetic background, the individual's
     initial plasma lipid concentrations and the varying
     responsiveness of the different classes of plasma lipid
     and lipoproteins, i.e., serum triglycerides appear to
     be more responsive than total cholesterol and chylo-
     microns more responsive than LDL under most conditions;

2.   Reducing dietary cholesterol mainly reduces the con-
     centration of total plasma cholesterol and LDL in
     normals and in those individuals with elevated LDL
     (Grundy, 1979; Connor and Connor, 1977; Anderson et
     al., 1973). Some studies also indicate that increase
     in dietary cholesterol consumption has an effect on
     HDL levels, although slight (Connor and Connor, 1977);

3.   Reducing dietary saturated fat reduces total
     plasma cholesterol, LDL and VLDL (Grundy, 1979;
     Connor and Connor, 1977; Brown, 1971). The hyper-
     or hypocholesterolemic effect due to the type of
     dietary fat is believed to be associated with the

particular type of fatty acid content. The satu-
rated fatty acids of less than 12 carbon atoms and
stearic acid ($C_{18:0}$) appear to have no influence,
while lauric ($C_{12:0}$), myristic ($C_{14:0}$) and palm-
itic ($C_{16:0}$) appear to have a direct influence
(Brown, 1971);

4. Ingesting large amounts of polyunsaturated fats in
the diet (in some studies as high as 20 percent of
the total calories) results in a fall in plasma
total cholesterol and LDL levels, and, to a lesser
extent, plasma triglycerides and VLDL, especially
in persons with elevated triglyceride concentrations
with a composition predominantly of saturated fat.
The absolute fall in plasma cholesterol concentra-
tions will vary depending on initial concentrations
and a responsiveness factor, but, in general,
persons with the highest initial plasma cholesterol
show the greatest decrements (Grundy, 1979).
Monounsaturated fats (i.e., oleic acid) have a
neutral effect, i.e., little or no effect on
plasma cholesterol concentrations. The cholesterol-
reducing effect of polyunsaturated fat is one-half
the cholesterol raising effect of saturated fats
(Anderson et al., 1973, Hegsted et al., 1965; Keys
et al., 1957a, 1957b);

5. "Carbohydrate-induced" hypertriglyceridemia, i.e.,
in studies where persons have been placed on a
high carbohydrate diet (above 65 percent of total
calories) from their usual mixed diet, there is a
rise in the plasma triglyceride level (Ahrens et
al., 1961). The degree of rise depends on whether
the person already has an underlying hypertriglyc-
eridemia. In most subjects, particularly those
with initially normal plasma triglyceride levels,
the "carbohydrate-induced" hyperlipidemia appears
to be a short-term phenomenon (Connor and Connor,
1977).

It is obvious from the above discussion that both the
quantity and quality of fat intake influences serum lipid and
lipoprotein levels. Most people express the quality of fat as the
amount of polyunsaturated to saturated fat in the diet, i.e., P/S
ratio. At the Cleveland Clinic, we introduced the concept of
"critical limit" which is the fatty acid composition of the diet
at which the change from partial to full effectiveness in serum
cholesterol reduction occurs (Brown, 1971). For example, critical
limits for a diet having 38 percent of calories as fat and 200 mg

of cholesterol intake, is 13 percent saturated fat and 14 percent polyunsaturated fatty acids, i.e. effective diets for reducing serum cholesterol levels contain up to 12 percent saturated fat and 14 to 22 percent polyunsaturates. With reduction in total dietary fat, these critical limits for the polyunsaturates are higher. The P/S ratio by itself may provide inadequate information. The critical limit concept accounts for the proportion of fatty acids in relation to total fat intake. Total calorie intake is important in its relation to body weight, i.e., hypertriglycerid- emia may result from caloric excess when it is accompanied by gain in body weight. This is of particular significance if the indi- vidual already has an elevated plasma triglyceride concentration. Overweight is also associated with elevated total plasma choles- terol concentrations caused in part by elevated VLDL (Connor and Connor, 1977).

Studies with various animal models also have shown similar findings. For example, adult, male ground squirrels were fed a rodent chow diet (4.5% total fat) supplemented with 2% cholesterol (by weight) for a year and no elevation in serum cholesterol or triglycerides was observed (Naito and Gerrity, 1979). Like other previous studies, the following experiment emphasized the role of fats in influencing the coefficient of absorption of dietary cholesterol and/or decreased cholesterol catabolism and excretion to cause an elevation in the serum cholesterol level. Feeding a diet high in fat and cholesterol (egg-yolk diet), resembling a typical American diet, produced hypercholesterolemia in this animal model (the ground squirrel). Although the cholesterol content of this diet was 50% less than the low-fat rodent chow diet fortified with 2% cholesterol, the high fat content (30.4% vs. 4.5%) may have increased the coefficient of absorption of dietary cholesterol. It is well known (Grundy, 1979) that the intestinal absorption of dietary cholesterol is influenced by the amount and type of fat in the diet and the physical state of the dietary cholesterol (i.e., crystalline vs. noncrystalline). The lower proportion of polyunsaturated fats vs. saturated fats (P/S ratio) in egg-yolk (P/S = 0.44) vs. that of the rodent chow (P/S = 0.81) may have had an additional effect on the observed mild hypercholesterolemia.

While the effects of dietary cholesterol in inducing hyper- cholesterolemia in laboratory animals are convincing, the dietary cholesterol effects in man are less clear. Certain animals such as the rat and dog are resistant to hypercholesterolemia by choles- terol feeding alone. The coefficient of absorption of these two animal models is high (i.e. about 80-90%) but their ability to completely suppress cholesterol synthesis and increase cholesterol catabolism and excretion is remarkably high, thus enabling them to maintain serum cholesterol homeostasis (Dietchy and Wilson, 1970).

The rabbit, on the other hand, readily absorbs cholesterol, but
seems to have difficulty in catabolizing and excreting exogenous
cholesterol. We have found that the prairie dog may fit into
this category since it, too, is extremely responsive to cholesterol
feeding (Holzbach et al., 1976; Naito et al., 1977). In man, the
coefficient of absorption is around 40%, depending upon the
composition of the diet and the amount of dietary cholesterol.
There is still some controversy as to how well the suppression of
cholesterol synthesis works by the negative feedback system in
man. It is believed that the 3-hydroxy-3-methyl glutaryl CoA
reductase, the rate-limiting enzyme for cholesterol synthesis,
can be suppressed by the end product negative feedback system,
but only partially in man (Grundy et al., 1969). Mattson et al.
(1972) have shown that by increasing the cholesterol content of a
diet originally low in cholesterol, the serum cholesterol concen-
tration increases by 5-12 mg/dl/100 mg dietary cholesterol/1,000
cal., providing all other dietary constituents remain unchanged.
When the original diet contains substantial amounts of cholesterol,
additional dietary cholesterol does not seem to greatly affect
the serum concentrations (Kummerow et al., 1977). However, not
all authors agree (Connor and Connor, 1977).

Plant sterols (i.e. campesterol, stigmasterol and β-sitos-
terol) are observed in the blood only to a slight extent, since
most of them are excreted in the stool. Of these phytosterols,
β-sitosterol usually predominates in the blood, constituting 50-
60% of the total plant steroids. In a typical American diet,
which may contain between 200-400 mg of β-sitosterol/day, its
coefficient of absorption is about 20-30%. In pharmacologic
amounts, β-sitosterol has been known to have a hypocholesterolemic
effect because of interference with the absorption of cholesterol.
Mattson et al. (1977) suggested that the extensive solubility of
the phytosterol esters in fat, in contrast to the limited solu-
bility of their unesterified form, provides a means for administer-
ing effective amounts of these hypocholesterolemic agents in
dietary therapy.

Purified cholesterol is quite unstable when stored in air at
room temperature. Recent data on the cytotoxic effects of prod-
ucts of cholesterol auto-oxidation (namely 25-hydroxycholesterol
and cholestane-3β, 5α, 6β-triol) on aortic smooth muscle cells
warrant mentioning (Imai et al., 1976; Peng et al., 1978), since
it is conceivable that the effects might lead to initial lesion of
atherosclerosis. Imai et al. (1976) showed that impurities in
old USP-grade cholesterol stored at room temperature caused aortic
lesions when a total of 1 gram of concentrated auto-oxidative
products of cholesterol/kg body weight was administered to rabbits
over a seven-week period. The possible implication of these
findings is that foods containing cholesterol, particularly those

that receive heat and oxygen treatment during processing (i.e. spray-dried eggs and whole milk) could prove to be hazardous to one's health. Their work further suggests that pure cholesterol (either endogenously synthesized by the animal or chemically isolated purified cholesterol) is not atherogenic. Only when cholesterol becomes a mixture of cholesterol plus spontaneously produced toxic derivatives does it become highly atherogenic. It appears that future studies should include an evaluation of commercially available foods that probably contain toxic derivatives of cholesterol.

Related to the cytotoxic effects of cholesterol auto-oxidation products is hypervitaminosis D. Vitamin D_3 feeding (intermittent or continuous) does not appear to influence serum cholesterol concentrations, but does result in development of atherosclerosis and calcified coronary arteries in animals with normal serum lipid concentrations (Kummerow, 1979). Kummerow et al. (1976) have noted the presence of cellular debris in the abdominal aortas of swine when given higher levels of vitamin D_3 than the NRC recommendations for swine. Additional findings suggest that high intake of vitamin D_3 will cause degeneration of smooth muscle cells in the aorta (Kummerow, 1979). This toxic substance is covered in greater detail in another chapter in this text.

Carbohydrates

In discussing carbohydrates, it becomes important to attempt to separate out effects of the different types of carbohydrate, i.e., complex carbohydrates (starch, polysaccharides) versus simple sugars. The literature in this area is controversial and inconclusive. Keys et al. (1960) observed that diets containing sucrose and lactose to provide 17 percent of total daily calories, produced significantly higher serum cholesterol concentrations in their schizophrenic subjects than diets in which an equivalent amount of carbohydrate was provided by a mixture of fruits, vegetables and legumes. Similarly, Antar and Ohlson (1965), comparing diets containing a 4-to-1 ratio of simple sugars to carbohydrate from potatoes, legumes, vegetables (as well as the reverse) demonstrated an increase in serum total lipids during ingestion of simple sugars. McGandy, et al. (1966), exchanging 23 percent of total daily calories between mono- and disaccharides and carbohydrate from potato and cereal, found higher plasma cholesterol concentrations when the subjects consumed the high simple sugars diet. However, other studies have not demonstrated any differential effect of simple sugars versus starch on plasma lipids (Irwin et al., 1964; Dunnigan et al., 1970). The work with patients with hyperlipidemias (hypertriglyceridemia, hypercholesterolemia, or mixed hyperlipidemia) suggests that isocaloric replacement of

sugar for starch adversely affects plasma lipids (Kuo and Bassett, 1965; Kaufmann et al., 1966), but for most persons, this effect occurs only in diets where saturated fat intake is relatively high, i.e., a low P/S ratio (0.1-0.3) and cholesterol intake about 600–800 mg, the usual American intake (Antar et al., 1970; Birchwood et al., 1970; Van Soest and Robertson, 1977).

Complicating the carbohydrate question (simple sugars versus starch) is that there may be significantly more dietary fiber in a high starch versus a high simple sugar diet. Thus, the effect of starch on cholesterol or triglyceride and/or lipoproteins may be a result of the level of dietary fiber in the test diet or the type of dietary fiber. Dietary fiber, i.e., cellulose, hemicellulose, pectins, mucilages, gums, lignin, represents the food components that cannot be digested in the upper gastrointestinal tract of nonruminants. In actuality, many studies report the crude fiber content of the diet and not the dietary fiber. The reason is that only crude fiber values for food are available in present food composition tables, and enough data is not yet available on dietary fiber content of most foods. The term "crude fiber" really represents an analytical technique for determining the dietary fiber of a food; it is the fraction remaining after food materials are treated with fat solvents, strong acid, and alkali. This technique destroys a portion of hemicellulose, cellulose, pectins and gums (Van Soest and Robertson, 1977). Much investigation to date has centered on the effects of crude fiber, or the "true" dietary fiber, in toto, or as separate components (pectin, lignin, cellulose) on serum lipid levels in normolipidemic or hyperlipidemic humans. Experimental results have been conflicting probably due to: a) differences in the type of dietary fiber used, b) use of crude fiber versus dietary fiber, c) amount and source of dietary fiber or crude fiber, and d) other components of the diet known to influence plasma lipids, i.e., cholesterol intake, saturated fat, and the initial plasma lipid levels of the subjects. We have found that the source of plant fiber is exceedingly important in determining its hypocholesterolemic effects (Malinow et al., 1976a, 1976; 1978). When cynomologus monkeys are fed wheat bran, rice bran or soya bran, no hypocholesterolemic effect is observed, while addition of alfalfa to the diet is not only hypocholesterolemic but reverses coronary atherosclerosis. Subsequent studies suggest that the alfalfa contains saponins that bind cholesterol in the gut, which prevents its uptake by the gastrointestinal tract – a mechanism different from that of other fiber effects, i.e., binding of bile acids. To date, in humans, it appears that, of the dietary fiber components: (a) bran has no effect on serum cholesterol or triglyceride concentrations (Connell et al., 1975; Eastwood, 1969; Jenkins et al, 1975; Truswell and Kay, 1975; Eastwood et al., 1973; Bremner et al., 1975), (b) large amounts of cellulose (60 to 100 g/d) may lower serum cholesterol

in persons ingesting diets with added excessive cholesterol
supplements (4 g) (Shurpalekar et al., 1971), (c) pectin (12 to
36 g/d) added to the diet produces a lowering of serum cholesterol
(Durrington et al., 1976; Keye et al., 1961) The degree of lipid
lowering may also depend on the amount of cholesterol in the diet
(Fisher et al., 1965). The mechanism(s) of the hypocholesterol-
emic effect of fiber have not been completely elucidated. There
are at least three possible mechanisms by which fiber may exert
its serum cholesterol lowering abilities:

1. saponin-cholesterol binding in the gut.
2. fiber-bile acid binding in the gut.
3. decrease cholesterol absorption due to increased
   transit time of the intestinal contents.

First described in 1918 (Feigle, 1918), alcoholic hyperlipemia
has been subject to extensive investigations over the past two
decades (Mistius and Ockner, 1972; Leiber et al., 1962; Schapiro
et al., 1965; Lifton and Scheig, 1978). Studies by Losowsky et
al. (1963), Schapiro et al. (1965) and Lifton and Scheig (1978)
demonstrated that alcohol ingestion in chronic alcoholics can
cause hypertriglyceridemia. This lipid abnormality is resolved
rapidly with abstention from alcohol and is apparently not provoked
by a high-carbohydrate diet. The sera of chronic alcoholics
frequently show a Type IV hyperlipoproteinemic pattern with high
levels of very-low-density lipoprotein and elevated triglycerides
and cholesterol. The alcohol-related Type IV hyperlipoproteinemia
appears different from the carbohydrate-induced Type IV and is
independent of acute pancreatitis, liver dysfunction, and obesity.
Often, normolipidemic individuals who have imbibed heavily on the
weekend will show marked hypertriglyceridemia lasting for several
days. It is not uncommon to observe concentrations in excess of
1,000 mg/dl in these individuals, particularly if they are hyper-
triglyceridemic subjects who are sensitive to caloric excess, causing
hyper-pre-β-lipoproteinemia (Naito, unpublished report). Bouchier
and Dawson (1964) suggested that alcohol might effect serum lipid
concentrations by the impairment of plasma triglyceride removal
and increased synthesis of triglyceride-rich lipoproteins. The
effects of alcohol vary and relate to dose, duration, the indi-
vidual's sensitivity, and initial blood lipid pattern. Ginsberg,
et al. (1974) have shown that the consumption of alcohol (three
ounces ethanol daily for seven days) by non-alcoholic, hypertri-
glyceridemic persons increases their fasting and postprandial
plasma triglyceride concentrations whereas in normal persons,
plasma triglycerides are increased postprandially only; total
plasma cholesterol and HDL-cholesterol levels were unchanged, LDL-
cholesterol decreased, and VLDL-cholesterol increased. In their
study, alcohol was added on to an already calorically-adequate
diet. When a large quantity of alcohol is consumed (up to 8 or 10

ounces of whiskey or the equivalent per day), plasma triglycerides
may increase transiently in normals or alcoholics (Connor and
Connor, 1977). We have found that a weak positive association
exists between daily alcohol intake and HDL-C (r = 0.15, p<0.001)
in the 800 male executives (50 ± 10 years) studied in the north-
east Ohio area (Naito et al., 1979). When compared to non- and
social drinking (3-4 drinks/wk) groups, moderate drinking (1-2
drinks/day) was associated with a slight elevation (6%) in HDL-C
(p 0.025), but total cholesterol (p<0.005) and LDL-cholesterol
(p<0.005) also rose, with no resultant change in the HDL-C/TC
ratio. Heavy drinking (>3 drinks/day) was associated with a 15%
elevation in HDL-C (p<0.001) and an increased HDL-C/TC ratio.
However, there were greater incidences of other risk factors, i.e.
elevated SGOT, blood pressure, body weight, smoking. Results of
our study are not in complete agreement with other published
reports. Although a weak association exists between alcohol
intake and HDL-C levels, other CHD risk factors appear with alco-
hol intake, suggesting that serum HDL-C levels alone do not com-
pletely explain the reported decreased incidence of CHD.

Proteins

        According to Connor and Connor (1977), the type of protein
consumed in mixed human diets has been shown to have no signifi-
cant effect upon plasma lipid levels in man. However, casein
appears to be more cholesterolemic and/or atherogenic than soya
protein for chickens (Kritchevsky et al., 1959) and rabbits
(Newburgh and Squire, 1920; Meeker and Kesten, 1940). Dietary
protein influences blood lipids, particularly cholesterol in
growing animals (Leveille and Fisher, 1958; Nishida et al., 1958;
Yen and Leveille, 1969). Further studies on dogs and rats that
have been fed a protein-free diet is presented in another chapter
of this monograph. In individuals with Kwashiorkor, there appears
to be decreased serum β-lipoprotein, total cholesterol, triglyc-
erides, and albumin, but α-lipoprotein and lecithin levels are not
reduced (Onitiri and Boyo, 1975). It has been suggested that the
decreased synthesis of the apoprotein moiety of LDL is the limiting
factor for the low lipids (Flores et al., 1970) in protein de-
ficiency. The impaired rate of synthesis of the lipoprotein would
cause triglycerides to accumulate in the liver. Human diets with
less than eight percent of calories as protein are associated with
low serum cholesterol (Brown, 1971) apparently due to the limi-
tation of protein and not caloric intake or not as a result of
lowered dietary fat intake.

NUTRITIONAL MODIFICATION FOR THE PREVENTION AND TREATMENT OF
HYPERLIPIDEMIA AND DYSLIPOPROTEINEMIA

With the development of the original concepts of the five
recognized types of dyslipoproteinemic conditions by Fredrickson
et al. (1967), five types of diets were formulated and prescribed.
The different diets have about the same fat composition when
available food products are used, except for the Type I diet.
All have less than 300 mg of cholesterol and are similar in their
ratio of polyunsaturated to saturated fat (P/S). Thus, a single-
diet concept for the treatment of hyperlipidemia is emerging that
should be simpler and more practical. Connor and Connor (1977)
suggested an "alternative diet," in which cholesterol intake is
100 mg/day and protein, carbohydrate and fat intake is 15, 65
and 20% respectively. The P/S ratio is 1.3 and crude fiber
intake of 12-15 g/day is recommended. The Inter-Society Commission
for Heart Disease Resources recommends the use of a moderate fat
diet, suggesting the use of 30-35% of the total calories as fat
(<10% saturated fat and >10% as polyunsaturated fat). Cholesterol
intake is maintained below 300 mg/day. Originally, the Cleveland
Clinic used two different diets (Brown, 1971). One was low in
fat, with 15% of the total calories as fat, and the other a
vegetable oil diet, with 38% of the total calories as fat. Serum
lipids were equally reduced with either diet in hypercholesterol-
emic subjects (Brown et al., 1973). In hypertriglyceridemic
patients(Types IIb and IV) serum lipids were reduced with greater
effectiveness with the 38% fat diet than with the 15% diet, i.e.,
reduction in carbohydrate intake. The Clinic then increased the
fat content of the Master Food Plan (semi-low-fat diet) from 33%
to 35%, with an elevation of the P/S ratio to 2.0 (Table 6). The
Master Food Plan may be used as a guide with which to commence
treatment of most patients with the various forms of primary
hyperlipoproteinemias. The fat content may be varied as best
suits a person's needs. The Master Food Patterns include a range
of fat-containing diets, from one with a very low fat content
(15%) but high in carbohydrates (65%) to one with a fat content
near that of the American diet (40%), but low in carbohydrates
(40%).

Patients with hypercholesterolemia will respond to a diet
with a fat content of 35% of the total calories and with a P/S
ratio of 2:1. The quality of the dietary fat is exceedingly
important. The intake of animal fat, which is generally high in
saturated fat should be minimized; about 7 ounces of low fat meats
(Table 7) a day is the suggested maximum. The polyunsaturated
fats (liquid vegetable oils, with the exception of coconut and
palm oils) should be substituted for animal fats in the diet.
Not all vegetable oils have the same quality of fat. Safflower
oil has the highest content of polyunsaturated fats (Table 8),
while coconut and palm oils have the lowest. Olive and peanut

Table 6.   Nutrient Composition of The Cleveland Clinic
           Master Food Plan[a]

Food Patterns	Fat % Calories	Carbohyd. % Calories	Prot. % Calories	P/S Ratio	Cholesterol (mg/day)
Typical American Diet	40	45	15	0.4	750
Master Food Plan	40	40	20	1.3	300
Semi-Low Fat Diet	35	50	15	2.0	100-300
Low-Fat Diet	15	65	20	1.3[b]	300

a
  Mean value
b
  The P/S ratio will vary from individual to individual, range =
  1.0 - 1.4

Table 7.   Classification of Meat, Fish, Poultry
           by Fat Content

Low Fat	Medium Fat	High Fat
Poultry	Roast	Bacon
Veal	Steaks	Salt Pork
Fish	Hamburger	Sausages
	Lamb	Duck
	Pork	Goose
	Ham	Luncheon Meats
		Frankfurters
		Beef marbled with Fat
		Chicken and Turkey Fat

Table 8. Fatty Acid Content of Some Food Products[a, b]

Items	Saturated Fatty Acids	Mono- Unsaturated Fatty Acids	Poly- Unsaturated Fatty Acids
Oils, Salad or Cooking			
Safflower	8	15	72
Corn	10	28	53
Soybean	15	20	52
Cottonseed	25	21	50
Sesame	14	38	42
Peanut	18	47	29
Olive	11	76	7
Coconut	86	7	--
Beef Products[c]			
Porterhouse	36	17	16
Sirloin	29	14	13
Round	12	6	5
Hamburger, lean	10.0	5	4
Hamburger, regular	21.2	10	9
Pork[c]			
Ham	10	11	2
Loin	25	9	10
Fish[c]			
Trout	3	2	--
Salmon	5	5	--
Chicken[c]			
Dark meat with skin	6	2	2
Dark meat without skin	4	1	1
Cheeses			
Cheddar	18	11	1
Cottage	2	1	--
Cream	21	12	1
Swiss	15	9	1
Butter	46	27	2
Margarine	18	47	14
Milk, whole	2	1	--
Egg, whole	4	5	1

a - In percent
b - From Watt and Merrill (1963)
c - Uncooked

Table 9. Cholesterol and Total Fat Content of Some
          Selected Food Items: Fish, Poultry, Meats[a, b]

Item	Chol/100 gm	Fat/100 gm
Haddock	60	0.5
Halibut	50	7.0
Salmon	47	5.9
Tuna	63	0.8
Sardines	140	8.6 (raw)
Shrimp	150	0.8
Scallops	53	1.4
Clams	63	1.6
Crab	101	1.9
Lobster	85	1.5
Oysters	50	1.8 (raw)
Chicken		
Light	79	3.4
Dark	91	6.3
Turkey		
Light	77	3.9
Dark	101	8.3
Beef	91	5.3
Liver	438	3.8 (raw)
Lamb	100	7.5
Pork	88	8.6

a - Lean (where possible) and cooked
b - From Feeley et al. (1972) and Watt and Merrill (1963)

oil may be used occasionally. Only special margarines containing
large amounts of unhydrogenated liquid vegetable oils are suitable
for the diet. Brown et al. (1973) have suggested that knowing
the P/S ratio is not sufficient in planning an effective diet
because it alone does not indicate the level of fatty acids.
When 36-42% of the calories come from fat, the "critical limit"
of saturated fat in the diet must be <14% and the polyunsaturated
fat must be >15%. In the semi-low fat diet, where 35% of the
calories are derived from fat, the maximum allowable amount of
saturated fat is 12% and the minimum polyunsaturated fat is 13%.
As can be seen, the "critical limits" account for the proportion
of fatty acids in relation to the total fat, which will vary from
one prescribed diet to another to suit each individual need.
Eggs should be limited to 2-3 per week. Careful attention should
be given to shellfish, sardines and liver for the high cholesterol
content (Table 9). Notice that crab and shrimp are also high in
cholesterol content. Liver contains five times as much cholesterol
as beef (steaks). Therefore, when adding liver to the diet,
careful attention should be given to the cholesterol and fat
composition. Type IIa hyperlipoproteinemic subjects, particularly
homozygous hyper-β-lipoproteinemic, may be very resistant to this
diet and require further reduction in cholesterol intake from 300
to 200 or even 100 mg/day, followed by drug therapy if necessary.

Hypertriglyceridemia and hyper-pre-β-lipoproteinemia (Type
IV) are reduced by a diet low in carbohydrate (Ahrens et al.,
1961) and require a carbohydrate intake of less than 50%, of
which the simple refined sugars should be substituted with complex
carbohydrates (starches, dietary fiber). The Cleveland Clinic's
basic moderate-fat diet calls for 40% of the total calories as
fat and carbohydrates. If hypertriglyceridemia and hypercholes-
terolemia (Type V) exist, the semi-low-fat diet should be utilized
to reduce the exogenous fat intake to 30% of the total calories
and the carbohydrate intake to 50%. The other two mixed hyper-
lipidemias (Types IIb and III) respond to the moderate-fat diet.
Table 10 gives suggestions on how the Master Food Plan may be
applied to the various dyslipoproteinemic conditions. It is
imperative that the proper diagnosis be made and that all acquired
disorders are corrected before instituting the prescribed diets.
It must be remembered that the caloric intake of the diets is
calculated on the basis of obtaining and maintaining ideal body
weight. Since the serum lipids and lipoproteins usually respond
to the prescribed dietary therapy within three weeks, a follow-up
program is necesary. Continuing encouragement and reassurance
are needed along with reliable information if the program is to
be successful.

When formulating a particular diet, it is important that a
conscious effort be made to decrease the simple sugar consumption
and replace it with complex carbohydrates. Consumption of fruits

and vegetables should be highly encouraged. At present, the
average American consumes about 2-4 g of dietary fiber daily,
while vegetarians consume about 24 g/day. Perhaps the dietary
fiber intake should be increased to about 10-15 g/day for adequate
nutrition. During the past century, composition of the average
American diet has changed; complex carbohydrates (fruits, vege-
tables, grain products), which once were the main-stay of the
diet, now play a minor role. At the same time, fat and sugar
consumption have risen markedly; these two dietary elements alone
now comprise more than 60% of our total caloric intake, up 50%
from the early 1900's (Dietary Goals for the United States,
1977). Table 11 provides examples of fiber content of selected
food items. The data was generated from the Highland View Hospi-
tals-Case Western Reserve University Data Base which has the
capability of analyzing more than 2,300 food items and recipes;
over 71 specific nutrients of each food item can be determined.

Since plant proteins have a plasma cholesterol lowering
effect as compared with animal protein, the diet should be com-
prised of at least 50% plant protein, as compared to 30% in the
standard American diet.

It is important that alcohol intake be restricted, especially
by the Type I and V patient, when following the prescribed diets.
Patients with the other dyslipoproteinemic forms may tolerate
alcohol if taken only occasionally and providing it does not
interfere with the weight reduction program or maintaining ideal
body weight.

Table 10 summarizes the use of the Master Food Plan for the
various hyperlipidemic and hyperlipoproteinemic conditions.

Since elevated blood pressure may accompany hyperlipidemia
in 15-20% of the patients, salt intake should also be monitored.
Salt (sodium chloride) consumption in the U.S. is estimated to be
between 6 and 18 g/day. The Select Committee on Nutrition and
Human Needs suggests reducing salt consumption by about 50-85%,
to approximately 3 g/day (Dietary Goals for the United States,
1977).

In the last analysis, it is preventive measures through
public education that will best enable us to achieve our ultimate
goal of minimizing the risk to atherosclerosis. It is particularly
important that the parents are educated so that proper behavior
patterns and preferences are taught to children by the age of
six, an age when acquired traits are probably well-implanted.

Table 10. Summary: Use of The Cleveland Clinic Master Food Plan For Primary Dyslipoproteinemias

Type of Hyper-lipidemia	Elevation of Lipoproteins	Type of Hyper-lipoproteinemia	Diet (P/S Ratio)
Exogenous Triglycerides	Chylomicron	I	Low Fat, 15% (P/S=1.3%)
Endogenous Triglycerides	Pre-β-	IV	Moderate Fat,40% (P/S=1.3%)
Cholesterol	β-	IIa	Semi-Low Fat,35% (P/S=2.0%)
Mixed Hyperlipidemia	β- Pre-β-	IIb	Moderate Fat,40% (P/S=2.0%)
		III	Moderate Fat,40% (P/S=1.3%)
		V	Semi-Low Fat,30% (P/S=1.3%)

Table 11.   Fiber Content of Selected Foods

	Amount	Fiber (gm)
Pear, raw with skin	1 medium	2.3
All Bran	1 oz.	2.1
Apple, raw with skin	1 medium	1.4
Watermelon, raw	1 wedge	1.3
Bran Flakes, 40%	1 oz.	1.0
Peanuts, roasted	1 oz.	0.77
Almonds, shelled	1 oz.	0.74
Wheat germ, dry	1 oz.	0.71
Orange, raw	1 medium	0.65
Shredded Wheat biscuit	1 oz.	0.65
Pecans, shelled	1 oz.	0.65
Applesauce, canned	1/2 cup	0.64
Walnuts, shelled	1 oz.	0.60
Banana, raw	1 medium	0.60
Corn, cooked (white/yellow)	1/2 cup	0.58
Carrot, raw	1/2 cup	0.55
Whole wheat bread	1 slice	0.45
Pineapple chunks, canned	1/2 cup	0.37
Celery, raw	1/2 cup	0.31
Pineapple, raw	1/2 cup	0.31
Tomato, raw	1/2 cup	0.31
Grapes, raw (Concord/Delaware)	12	0.29
Raisins	2 tbsp.	0.16
Lettuce, iceberg	1/2 cup	0.14
Orange juice, canned	1/2 cup	0.12
Apple juice, canned	1/2 cup	0.12
Rye bread, light	1 slice	0.11
Corn Flakes	1 oz.	0.09
White bread, enriched	1 slice	0.05
Oatmeal or Rolled Oats, cooked	1 oz.	0.05

a
  From Highland View Hospital - Case Western Reserve University
  Nutrient Data Base, Departments of Nutrition and Biometry, Case
  Western Reserve University, Cleveland, Ohio 44106.

ROLE OF THE PHYSICIAN, CLINICAL BIOCHEMIST AND DIETITIAN
IN DIETARY MANAGEMENT

The ideal approach in attempting to lower a patient's risk
for CHD is a team approach, particularly when considering dietary
management for treating hyperlipidemia. An intimate working
relationship should exist between the physician(s), clinical
biochemist, dietitian and patient. It is imperative that the
physician and clinical biochemist recognize the importance of
nutrition in the overall health care of the patient, have a full
understanding and working knowledge of nutritional factors that
have been associated with high risk for CHD, and have current
knowledge on the most effective way(s) to control the lipid
abnormalities via proper dietary management. In the development
of this intimate relationship, the first step is close communication
with each other. The physician and clinical biochemist must
adopt the attitude that they can depend upon the professional
knowledge and experiences of the dietitian in helping him achieve
the goal of effective patient care. The dietition should be as
familiar with the patient as is the physician. Information on
the patient's life style, eating habits, likes and dislikes, food
practices, and CHD risks should be apparent to both the physician
and dietitian in order that they can plan the most effective
means of reducing the patient's risk to CHD. With this approach,
the responsibility of the dietitian should not only encompass the
control of elevated serum lipids through dietary management, but
should also include the consideration of diet in the control of
elevated blood pressure and body weight, and advice concerning
excessive smoking and lack of physical activity as a compre-
hensive health care therapy for the patient.

For this system of therapy to succeed, it is important that
the patient be informed of the seriousness of his physical condition
and the logic behind instituting the various approaches to mini-
mize the associated risk factors for premature coronary events.
Patient education is sadly lacking in most institutions. Unless
the physician, clinical biochemist and dietitian are confident,
knowledgeable and enthusiastic about the effectiveness of mini-
mizing the risk of CHD through dietary management, the patient
will not develop the attitude necessary to comply with the
prescribed diets and possible altered life style. During in-
struction, it is most important that the patient help in planning
his diet; the person buying the food and preparing the meals
should also be present. While hyperlipidemia can be genetically
determined, in many instances it is also due to environmental
conditions, especially improper food habits. If the latter
condition applies to the patient, the acquired hyperlipidemic
condition of the patient can also be potentially manifested in
the other members of the family, particularly the children.

Table 12.   The Nutritional Factors Influencing
            Serum Lipoproteins

Nutritional Factors	VLDL	LDL	HDL
Total Calories	↑	(↑)	(↓)
Total Fat	↑	↑	↓
Saturated	↔	↑	(↓)
Monounsaturated	↔	↔	↔
Polyunsaturated	↔	↓	(↑)
Cholesterol	(↑)	↑	(↑)
Phytosterols	↔	↓	↔
Protein			
Vegetable	?	↓	(↑)
Animal	?	↑	↔
Carbohydrates			
Simple	↑	↔	↓
Complex	↔	↓	↔
Alcohol	↑	(↑)	↑
Vitamin C	↔	↔	↔

Therefore, nutritional education directed toward all members of the family, particularly those who purchase and prepare the meals is beneficial to all. The prepared meals should, ideally, include the rest of the family members, unless the prescribed diet is drastic and applies only to a person with severe risk factors. It is our experience that group instruction is more effective than individual instruction because of the beneficial effects of interaction among members of the group.

One of the reasons for the poor adherence to diets is the poorly designed or even the lack of design of a follow-up period. This is important for both the physician and patient. The physician needs to monitor the blood lipids and lipoproteins to determine the effectiveness of the prescribed diet, while the patient needs further instructions, information on his progress, and positive reinforcement. In the follow-up period, visits at 2, 4 and 6 weeks after the initial two to three hour instruction should be adequate. After the initial follow-up period, less frequent visits (perhaps once a year) are required to insure adherence to the diet pattern. The continued objective is to follow through with the measurements that test adherence to the diet, including: body weight, blood lipids, lipoproteins and blood pressure. The examination should also include discussion of alcohol consumption and smoking patterns. Finally, the patient must always receive encouragement to stay on his diet and physical exercise program.

SUMMARY

In summary, the relationship of serum lipids and lipoproteins to CHD has been reviewed. The effect of specific nutritional components in the diet on serum lipid and lipoprotein concentrations has been discussed (and is summarized in Table 12). An approach to dietary management of hyperlipidemic and hyperlipoproteinemic conditions was provided as a basis for future dietary trials. Finally, the role of the physician, clinical biochemist and dietitian in dietary management of the CHD patient has been covered to emphasize the need of a team approach to patient care.

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DIETARY MANAGEMENT OF HYPERLIPIDEMIA: THE PRUDENT POLYUNSATURATED

FAT DIET

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INTRODUCTION

One of the most dramatic and exciting findings in recent years
in the scientific literature is the observed decline in coronary
heart disease (CHD) mortality rates in the United States. This
has been a steady phenomenon seen in white and non-white, men and
women since 1968, which included the entire age span from 35-74
years (Stammler, 1979).

Correspondingly, several trends have been proceeding in
parallel over these years:

1. Positive developments in emergency care techniques of
    CHD patients (both acute and chronic).

2. Improvements in early detection methods for the poten-
    tial CHD candidate.

3. Better equipped and staffed coronary care units.

4. Improvements in cardiac assist instrumentation.

5. More effective antiarrhythmia, antihypertensive and
    cardiopulmonary drugs.

6. Increased interest in physical exercise, better nutri-
    tion, decreased smoking and other behavior modification
    in controlling major risk factors.

7.   More wide-spread education of the general population on
     medical information.

There is reason to believe that diet modification for the
control of hypertension and hyperlipidemia has played an important
role in reducing the incidence of CHD (Stammler, 1979). Numerous
diet plans have been suggested by physicians, associations, and
expert committees over the past 20 years to decrease CHD risk. No
less than 20 expert scientific committees have recommended dietary
modification as a means for controlling blood lipid levels and
reducing the risk of CHD. Table 1 summarizes their recommendations.
Some countries, such as Canada, have adopted these recommendations
as a national policy.

The Select Committee on Nutrition and Human Needs has recom-
mended dietary goals for the American general population (Select
Committee on Nutrition and Human Needs, 1977). Although the diet
recommendations differ in some details, the basic fat-modified
diet as suggested by the Inter-Society Commission for Heart Disease
Resources is the common base; it is essentially a calorie controlled,
moderately low cholesterol and moderately low saturated fat diet.
It is intended to provide all essential nutrients in ample amounts
and to fit well into the life styles of industrialized, relatively
sedentary populations.

Turning our attention to the high-risk group for heart
attacks, how does one effectively use dietary means to control
CHD risk factors? The objective of nutritional modification is to
lower serum lipids and low-density lipoproteins sufficiently so
that hyperlipidemia and hyperlipoproteinemia as risk factors in
the development of CHD are eliminated or greatly reduced. Clearly,
the most important criteria of the therapeutic success in the use
of these diets are the long term serum lipid and lipoprotein
responses obtained in the individual patient.

What is needed is a detailed description of how therapeutic
diets for hyperlipidemia are formulated and written. How is a
diet developed for patients with hyperlipidemia? What are the
necessary tools when constructing a therapeutic diet? The follow-
ing is a detailed discussion of the developmental procedure of the
Cleveland Clinic Foundation's Prudent Polyunsaturated Fat Diet
(PPFD). This chapter will include the parameters of the diet, why
they were chosen, tools needed to develop a diet, the dietary
considerations needed to enhance patient adherence and patient
education.

Table 1. Recommendations of Expert Committees on Moderate Dietary Change to Reduce Risk of Coronary Heart Disease

Country and organization of origin	For gen. populations (GP) or high risk (HR) Groups	As % of Total Calories			Chol. (mg/day)	Decr. Sugar
		Fat	PUFA	SFA		
Scand. countries, 1968 Official Collective Recommend.	GP	25-35	Increase	Decrease	-	Yes
Un. States, 1972 Inter-Soc. Commission for Heart Dis. Resources	GP HR	<35	up to 10	<10	<300	-
Un. States, 1972 Am. Health Fndn.	GP	<35	10-12	10-12	300	Yes
Un. States, 1972 Am. Med. Assoc. & Nat. Acad. of Sciences	HR	Substan. decrease in sat. fat	Substit. PUFA for SFA	Decrease	Reduce	-
Internat. Soc. of Cardiol. 1973	HR	<30	-	Decrease	<300	Yes
The Netherlands, 1973 Nutrition Council	GP	30-35 Ideally 33	10-13	Decrease Substan.	250-300	Yes
Australia, 1974 Nat. Heart Fndn.	GP HR	30-35	Increase	Decrease	<300	-
United Kingdom, 1974 Dept. of Health & Social Security	GP	Reduce	-	Reduce	-	-
Germany, 1975	GP	20-25	10-15	Reduce	300	-
New Zealand, 1976	GP	35	Subst. PUFA for SFA	Decrease	300-600	Yes

Table 1. Recommendations of Expert Committees on Moderate Dietary Change to Reduce Risk of Coronary Heart Disease (Continued)

Country and organization of origin	For gen. populations (GP) or high risk (HR) Groups	As % of Total Calories			Chol. (mg/day)	Decr. Sugar
		Fat	PUFA*	SFA*		
U.K., 1976 Royal Coll. of Physicians & Br. Cardiac Soc.	GP	Toward 35	Subst. PUFA for SFA	Reduce	Reduce	Yes
Norway, 1976 Ministry of Agriculture	GP	<35	Increase	Decrease	-	Yes
Canada, 1977 Dept. of Nat. Health & Welfare	GP	30-35	Subst. PUFA for SFA	Decrease Substan.	<400	Yes
Ireland, 1977 An Foras Taluntais	GP	Reduce	-	-	-	-
Un. States, 1977 Dietary Goals	GP	30	up to 20	<10	<300	Yes
Un. States, 1978 Am. Heart Assoc.	GP	30-35	up to 10	<10	<300	-
Europ. Soc. of Cardiol., 1978	HR	Toward 30	Subst. PUFA for SFA	10	<300	Yes
Food and Agriculture Organ./ World Health Organ., 1978	GP	30-35	10-12	Decrease	<300	Yes

PUFA = polyunsaturated fatty acid; SFA = saturated fatty acid; Chol. = Cholesterol; decr. = decrease

RATIONALE OF THE DIET PARAMETERS

The initial dietary treatment for hyperlipidemia should meet the following criteria for maximum effectiveness:

1. Reduce cholesterol intake to ∿300 mg/day.

2. Increase polyunsaturated fat to saturated fat (P/S) ratio to 1.0-1.3.

3. Reduce total fat intake to 80-100 gms/day (35-40% of total kilocalories).

4. Maintain caloric intake between 1800-2500 kilocalories/day (applies to the average adult, doing normal routine work).

5. Limit sodium intake to 4-6 gms/day.

6. Decrease consumption of simple sugars.

It is generally recognized that CHD is caused by a combination of factors. Some of the known risk factors for CHD are high concentrations of blood lipids, hypertension, smoking, overweight, imbalanced and excessive food intake, diabetes mellitus, gout and renal disease, as discussed in the previous paper by Naito. Some of these risk factors can be modified or controlled by altering certain dietary habits. Some of these modifications include an increase in fiber intake, a decrease in the amount of dietary sodium, and a reduction of calorie intake until ideal body weight is achieved. Almost all lipids and lipoprotein disorders can be altered through appropriate diets (Keys, et al. 1965).

According to the American Heart Association (1973), the average American diet contains approximately 600 mg of cholesterol/day and 145 gms of fat with a P/S ratio of about 0.3. Consumption of this quantity and quality of fat is considered to be unhealthy for the general public, let alone for the high risk CHD individual with hyperlipidemia and hyperlipoproteinemia.

The Cleveland Clinic Foundation's Prudent Polyunsaturated Fat Diet was designed as a therapeutic regimen for hypercholes-terolemia and concentrates on Brown's (1976) Master Food Plan which utilizes lean meat, fish and poultry, low fat or skim milk dairy products and polyunsaturated oils and margarines (see Table 2).

The PPFD can be modified to be used for the dietary management of those individuals with elevated triglycerides and/or who

Table 2. Master Food Patterns[a,b,c]

Food Pattern	Fat %Cal.	CHO[d] %Cal.	Sat. F.A.[d] %Cal.	Poly F.A.[d] %Cal.
Basic Low Fat*	12	73	5	1
Moderate Low Fat*	20	65	5	8
Moderate Fat*	33	52	7	12
Customary Fat*	38	47	10	15

a – Mean daily protein intake: 15% of total calories
                                  (range = 15-20%)
b – Mean daily cholesterol intake: 300 mg (range = 75-300 mg)
c – Adapted from Brown (1976)
d – Carbohydrate, saturated fatty acids, polyunsaturated
    fatty acids

are above their ideal body weight. Since obesity is correlated to
a high consumption of refined, simple sugars, alcohol and an
excessive caloric intake (Kannel, et al. 1979), it seems prudent
to design a diet that controls these factors. Often a reduction
in dietary cholesterol and saturated fat result in a deficit of
kilocalories. For those individuals at their ideal body weight,
this deficit is supplemented with an increased intake of complex
carbohydrates such as starches, fruits, vegetables and vegetable
proteins.

The sodium intake as salt and high sodium foods can affect
the control of hypertension. The average daily diet of Americans
contains approximately 7-15 gms of sodium (Select Committee on
Nutrition and Human Needs, 1977). However, these figures will
vary depending on the quantity of convenience food items used in
the diet. The sodium content of these foods is available in a
handbook published by the West Suburban Dietetic Association
(Rezabek, 1979). When food selection is derived according to the
Basic Four Food Groups, the Recommended Daily Allowances (National
Research Council, 1974) are met for protein, vitamins and minerals.
The PPFD was designed with the above considerations in mind.

DEVELOPMENT OF THE THERAPEUTIC DIET

Certain basic considerations must be met when planning a
therapeutic diet. They include a profile of the patient providing
age, socioeconomic conditions, number of meals eaten away from
home per day, physical activity and limitations and the presence
of any chronic illness which may necessitate additional dietary
modification, i.e. diabetes mellitus, gout, hypertension. Diet
histories and a 24-hour recall (Figures 1-3) are tools that are
used in obtaining food preferences and details of an individual's
eating pattern. Setting up a summary containing this information
will aid the dietitian in planning a therapeutic diet that is
adaptable to an individual's life-style, needs and preferences.
This information will ensure optimal nutritional care, enhance
patient dietary adherence and provide a base for individual
instructions.

What tools are available to the dietitian when planning a
therapeutic diet? Reliable information on nutrients can be
obtained from food composition handbooks such as Church and
Church (1975) and the United States Department of Agriculture
(USDA) Handbook #456 (Adams, 1975), or from Handbook #8 (Watt and
Merrill, 1963), or a nutrient data bank. Food values are listed
in either grams or in common household weights and measures.
Access to a nutrient data bank greatly facilitates the dietitian's
work load when developing individually tailored diets.

The data bank at Case Western Reserve University is a com-
puterized food table data storage facility which contains more
than 2,300 food items and recipes. The food items, including
brand name items, in the data base have been selected as a result
of analysis of several thousand 24-hour intake records from
several areas of the United States. For each food item, there is
a storage space for 71 nutrients. (Table 3) The nutrient compo-
sition of foods is derived from a variety of sources (Adams,
1975; American Home Economics Association, 1975; Anderson, 1976;
Anderson et al., 1977; Anderson et al., 1975; Brignoli et al.,
1976; Dicks, 1965; Diem and Lentner, 1973; Dunbar and Stunkard,
1979; Ehrheart and Mason, 1967; Exler et al., 1977; Exler and
Weihrauch, 1976; Exler and Weihrauch, 1977; Feeley et al., 1972;
Feeley and Watt, 1970; Fetcher, 1967; Fristrom et al., 1975;
Fristrom and Weihrauch, 1976; Gormican, 1970; Hardinge, 1961;
Hardinge, 1965; Leung, 1961; Leverton and Odell, 1959; Marsh et
al, 1977; Matthews and Garrison, 1975; Mattice, 1950; McCarthy,
1968; Murphy et al., 1975; National Research Council, 1974;
Nutrient Composition of Common Sizes and Measures of Foods, 1970;
Organization of the United Nations, 1970; Orr, 1969; Orr and
Watt, 1957; Osis, 1972; Perloff and Butrum, 1977; Posati et al.,
1975; Positi et al., 1975; Positi and Orr, 1976; Streiff, 1971;

THE CLEVELAND CLINIC FOUNDATION
NUTRITION PROFILE FORM

Name:_____ Age:_____Height:____Weight:_____

Address:_____ Occupation:_____

_____ Scheduled Work Hours:_____

Phone:(home)_____

(work)_____ _____ Date:_____

1. List other household members:

NAME	RELATION	AGE	SPECIAL DIET FOLLOWED
a.			
b.			
c.			
d.			
e.			

2. Do you now or have you ever followed a "special diet"? Yes_____No_____

If yes, what type of diet?_____

How long did you follow the diet?_____

For what reason did you follow the diet?_____

From what source did you recieve the information about your diet?

Physician_____Dietitian_____Book or Magazine_____Title_____

Special diet of health food store_____Other_____

3. Do you take vitamin/mineral/diet supplements? Yes_____No_____

If yes, list:_____

_____

4. Who is responsible for home food preparation?_____

5. Who is responsible for food purchasing?_____

6. Which meals are most often eaten away from home:_____

Breakfast_____Lunch_____Dinner_____Snacks_____

7. Do you have trouble chewing or swallowing food Yes_____No_____

If yes, explain:_____

_____

8. Do you have any food allergies? Yes_____No_____

If yes, explain:_____

_____

Fig. 1. The Cleveland Clinic Foundation Nutrition History Form

NUTRITION PROFILE FORM - Page 2

9. Do you have frequent occurrences of the following?

	Yes	No			Yes	No
a. indigestion	___	___	d. diarrhea		___	___
b. nausea	___	___	e. constipation		___	___
c. vomiting	___	___				

10. Have you had any change in weight within the: a) past 3 months?
    b) past year? c) past 2 years? d) past 5 years? (please circle).
    If yes, explain:_____

    _____

    _____

11. Do you exercise or participate in athletic activities? Yes____No_____
    If yes, what type?_____
    How often?_____

12. What do you usually eat? Please list all foods that you usually eat
    between the time that you get up in the morning and the time you to to
    bed at night. Remember, everything you eat or drink is food. Cookies,
    nuts, sweets, salad dressings, gravy, cocktails, highballs, beer and
    soft drinks are food items just as are meat and potatoes. Indicate
    how the foods are prepared, such as broiled, baked, fried, boiled, etc.

At Breakfast	At Lunch	At Dinner
Mid-Morning Snack	Mid-Afternoon Snack	Before Bed Snack

During the night:_____

_____

Fig. 2. The Cleveland Clinic Foundation Nutrition History Form

NUTRITION PROFILE FORM - Page 3

Please indicate below how often you eat or drink the following items.
Include amount and where indicated, circle the type of food eaten.
Example Milk: whole   skim   low fat.

FOOD ITEM

Milk: whole   skim   low fat   chocolate:_____
Cheese   buttermilk   yogurt:_____
Pudding   cottage cheese   custard   ice cream:_____
Beef   lamb   pork: baked   broiled   fried:_____
Chicken   Turkey   veal: baked   broiled   fried _____
Fish   shellfish: baked   broiled   fried_____
Bacon   sausage   luncheon meat   hot dogs:_____
Liver   organ meats (kidney, tongue, etc.):_____
Eggs   egg substitutes:_____
Dried beans   peas   legumes:_____
Peanut butter   nuts   seeds:_____
Cereals: whole grain   sugar coated   other:_____
Breads: whole grain   white   other _____
Potatoes: baked   mashed   fried_____
Rice   macaroni   noodles   spaghetti:_____
Dark green   or   yellow vegetables:_____
Other vegetables (including lettuce):_____
Citrus fruit or juice (not fruit drink):_____
Other fruit or juice: sweetened   unsweetened_____
Cake   cookies   doughnuts   pastries   pie   "Jello":_____
Sugar   jelly syrup   honey   hard candy   chocolate candy:_____
Pretzels   potato chips   peanuts   salty snack foods:_____
Soups: cream   other_____
Butter   lard   shortening   bacon fat:_____
Margarine   vegetable oil (list brand names):_____
Salad dressings   mayonnaise:_____
Gravy   cream sauces:_____
Alcoholic beverages: Type beer/wine   other alcohol:_____
Kool-Aid   fruit drinks   lemonade   punch:_____
Soft drinks ("pop")   Diet soft drinks:_____
Coffee   tea   decaf. coffee   Postum:_____

Which of the following do you add to your coffee or tea? Indicate amount
per cup:

Nothing_____   Cream_____   Nondairy Cream_____   Sugar_____

Saccharin_____   Honey_____   Other_____

Fig. 3.   The Cleveland Clinic Foundation Nutrition History Form

Table 3. Nutrients - In HVH-CWRU Nutrient Data Base

Calories	Minerals and Trace Elements
Total Protein	Iron
Animal Protein	Calcium
Plant Protein	Phosphorous
Total Fat	Sodium
Animal Fat	Potassium
Plant Fat	Magnesium
Total Carbohydrate	Chlorine
Refined Carbohydrate	Chromium
Natural Carbohydrate	Cobalt
Alcohol	Copper
Ash	Iodine
Fiber	Manganese
Water	Molybdenum
Caffeine	Selenium
	Sulphur
Cholesterol	Zinc
Fatty Acids	
Total Saturated	Amino Acids
Total Unsaturated	Cysteine
Oleic	Cystine
Linoleic	Histidine
	Isoleucine
Vitamins	Leucine
Vitamin D	Methionine
Total Vitamin A	Phenylalanine
Performed Vitamin A	Threonine
Beta-Carotene	Tryptophan
Total Tocopherol	Tyrosine
Alpha Tocopherol	Valine
Other Tocopherol	
Ascorbic Acid	Sugars
Thiamin	Glucose
Riboflavin	Fructose
Niacin	Lactose
Pyridoxal B6	Maltose
Vitamin B12	Sucrose
Folic Acid	Reducing Sugars
Pantothenic Acid	
Biotin	
Choline	

HVH-CWRU = Highland View Hospital-Case Western Reserve University

Sturderant et al., 1973; Toepfer et al., 1951; Umbarger, 1965; Watt and Merrill, 1963; Weihrauch et al., 1976; Zook and Lehmann, 1968). All analytical data were substantiated and verified for appropriate analytical methodology and evaluated for accuracy by research dietitians and an experienced technical assistant before incorporation into the data base. Manufacturers' data were accepted only when they were received directly from the manufacturers and based on their chemical analyses. Data were subjected to several validation processes including programmed verification before insertion in the table. The data base is reviewed and updated on a continuing basis with the addition of new items or nutrient composition values as information becomes available. For many nutrients, the only available data are from the various scientific journals. This information is carefully evaluated for methodology and reliability before incorporation into the data base. It must be recognized that for some nutrients, i.e. Vitamin D, Vitamin B_6, iodine, copper, biotin and pantothenic acid, only a limited amount of reliable food composition data are available. All nutrient data for food items are calculated on the basis of 100 gram portions. However, analysis for all food items may be retrieved in a variety of weights (i.e. from grams to ounces or pounds and, for many items, analysis may be retrieved for volume equivalents and for descriptive terms). An example of a computer printout is illustrated in Figures 4-6.

NUTRIENT ANALYSIS SUMMARY
NAME:
HVH-CWRU NUTRIENT DATA BASE   DATE: Friday, November 30, 1979   ID: CC 002 05

			Kcal	T-Pro Gm	T-Fat Gm	T-CHO Gm	Chol mg	PUFA Gm	SFA Gm	Fiber Gm
Breakfast										
6	Vol Oz	ORANGE JUICE UNSW, CANNED	90	1.5	0.4	20.9				.1867
1	Large	EGG WHOLE, POACHED	79	6.0	5.6	0.6	273	.72	1.665	0
2	Wt Oz	CANADIAN BACON, BROILED OR FRIED, DRAINED, SERVING WT	157	15.7	9.9	0.2	50	1.527	3.365	0
1	Slice	WHOLE WHEAT TOAST	55	2.4	0.7	10.8			.1121	.361
2	Teasp	MARGARINE REGULAR, FLEISCHMANN	68	0.1	7.6	0.0	0	2.026	1.231	
Lunch										
2	Wt Oz	BEEF ROUND RUMP ROAST, BONELESS LM ROASTED, SERVING WT	118	16.5	5.3	0.0	52	.681	2.213	0
2	Slice	WHOLE WHEAT BREAD	112	4.8	1.4	21.9			.2714	.736
.5	Cup	PINEAPPLE CHUNKS, CANNED, SOLIDS & LIQUIDS, JUICE PACK	71	0.5	0.1	18.6				.369
8	Vol Oz	SKIM MILK	86	8.4	0.4	11.9	5	.0171	.2867	0
.33	Cup	SUGAR GRANULATED, WHITE	244	0.0	0.0	63.0	0	0	0	0
.167	Teasp.	CINNAMON, GROUND	1	0.0	0.0	0.3	0	.0020	.0024	.0921
.5	Cup	WALNUTS ENGLISH (HALVES)	326	7.5	32.0	7.9	0	21	3.47	1.05

Fig. 4. Example of the HVH-CWRU Nutrient Data Base Computer Printout.

			Kcal	T-Pro Gm	T-Fat Gm	T-CHO Gm	Chol mg	PUFA Gm	SFA Gm	Fiber Gm
Dinner										
4	Vol Oz	APRICOT NECTAR, VITAMIN C ADDED	72	0.4	0.1	18.3				.251
3	Wt Oz	FLOUNDER BAKED, SERVING WT	119	25.5	1.1	0.0	43	.5022	.2384	0
.5	Cup	POTATO PARED, BOILED & DICED OR SLICED	50	1.5	0.1	11.2				.3875
.5	Cup	WHITE SAUCE (MEDIUM)	203	4.9	15.6	11.0	16		8.75	
.75	Cup	BEANS GREEN, CUT STYLE CANNED, DRAINED SOLIDS	24	1.4	0.2	5.3				1.013
1	Slice	WHOLE WHEAT BREAD	56	2.4	0.7	11.0			.1357	.368
.5	Cup	CABBAGE RAW, COARSELY SHREDDED OR SLICED	8	0.5	0.1	1.9				.28
2	Teasp	MAYONNAISE	67	0.1	7.5	0.2	7		1.307	
2	Teasp	MARGARINE REGULAR, FLEISCHMANN	68	0.1	7.6	0.0	0	2.026	1.231	
Evening Snack										
1	Cup	Skim Milk	86	8.4	0.4	11.9	5	.0171	.2867	0
.5	Medium	BANANA YELLOW RAW, EP	51	0.7	0.1	13.2				.2975
6	Number	STRAWBERRIES, WHOLE FRESH UNSW	41	0.8	0.6	9.4				1.453

Fig. 5.   Example of the HVH-CWRU Nutrient Data Base
          Computer Printout

NUTRIENT SUMMARY

			% RDA	% CAL
Kilocalories	2250		83 %	
Total Protein	109.8	Gm	196 %	20 %
Total Fat	97.4	Gm		39 %
Total Carbohydrate	249.6	Gm		44 %
Cholesterol	449	mg *		
Total Polyunsat FA	28.52	Gm *		
Total Saturated FA	24.56	Gm *		
Fiber	6.844	Gm *		
Ascorbic Acid	252.6	mg *	561 %	
Thiamin	1.724	mg *	153 %	
Niacin	15.26	mg *	264 %	
Riboflavin	1.788	mg *	132 %	
Pyridoxal B6	1389	ug *	69 %	
Vitamin B12	2.469	ug *	82 %	
Folic Acid	.3154	mg *	79 %	
Total Vitamin A	4853	IU *	97 %	
Vitamin D	199.9	IU *		
Iron	15.72	mg	157 %	
Calcium	1105	mg	138 %	
Phosphorus	1820	mg	228 %	
Magnesium	424.1	mg *	121 %	
Copper	1.347	mg *		
Zinc	13.64	mg *	91 %	

HVH-CWRU Nutrient Data Base

Fig. 6.   Example of the HVH-CWRU Nutrient Data Base
          Computer Printout

The computer system of the Nutrient Data Base is a Digital
Equipment Corporation PDP-11/45 minicomputer running under the
UNIX operating system. The system is a 16-bit computer with 124K
words of core and semiconductor memory and 100 MB of on-line
secondary storage.

Information on food composition and nutrient adequacy through
the use of computers is a growing, time saving and valuable tool
for the dietitian. If a computer is not available the menus
developed from the designed diet will have to be hand calculated
to determine nutrient adequacy.

After the parameters of the diet have been established,
educational materials should be developed. The materials should
emphasize a positive approach to dietary change and why the
change is recommended. Written diet instruction materials can be
constructed using various formats. Having peers or patients
review the proposed material will help to ensure that the infor-
mation was covered in an understandable, comprehensive and accurate
way. The following format outlines the diet instruction material
used at The Cleveland Clinic Foundation:

1.   Introduction.

2.   Definition of key terms, i.e. cholesterol, saturated
      fat and polyunsaturated fat.

3.   Summary of the key principles of the diet.

4.   Discussion of food groups.

5.   Special instructions on label reading, cooking tips,
      shopping tips, dining out in restaurants and cookbook
      references.

Audiovisual aids and slide presentations can help to supple-
ment the written materials. It has been our experience that the
most effective way to teach the PPFD is in a classroom type of
environment. This method of teaching promotes participation from
patients as well as from their family members. Often patients who
are already familiar with the diet offer helpful suggestions on
meal preparation and shopping suggestions to those who are re-
ceiving their initial dietary instructions. A short slide presen-
tation emphasizing the key principles of the PPFD is also provided
in the instruction. This presentation leads into a group dis-
cussion monitored by the dietitian. Feedback regarding the effec-
tiveness of our diet class and patient compliance will be dis-
cussed later in this paper.

After the parameters of the therapeutic diet were established, The Cleveland Clinic Foundation's Prudent Polyunsaturated Fat Diet was developed by manipulating the use of saturated fat, polyunsaturated fat and cholesterol content of meat products, dairy products, margarines and oils (Table 4). The use of 2 egg yolks per week was calculated on a weekly basis (71 mg cholesterol/ day). To achieve the indicated P/S ratio, special consideration was placed on margarines. The PPFD recommends using margarines that list either liquid safflower, liquid sunflower, or liquid corn oil as the first ingredient and that the ratio of polyunsaturated fat to saturated fat is 2.0 (Figures 7 and 8).

NUTRITION INFORMATION PER SERVING

SERVING SIZE	1 TABLESPOON (14 GRAMS)
SERVINGS PER CONTAINER (ONE POUND)	32
CALORIES	100
PROTEIN	0 GRAMS
CARBOHYDRATE	0 GRAMS
FAT	11 GRAMS
PERCENT OF CALORIES FROM FAT†	99%
POLYUNSATURATED†	**4 GRAMS**
SATURATED†	**2 GRAMS**
CHOLESTEROL†	(0 MG./100 GM.) 0 MILLIGRAMS
SODIUM	(815 MG./100 GM.) 115 MILLIGRAMS

INGREDIENTS: **MAZOLA LIQUID CORN OIL**, Partially Hydrogenated Soybean Oil, Skim Milk, Salt, Lecithin, Monoglyceride, Natural Flavor, Artificial Flavor and Color (Carotene). Idopropyl Citrate and Calcium Disodium EDTA Added to Protect Flavor. Vitamins A and D added.

Fig. 7. Example of an unacceptable margarine. Note that the primary ingredient is liquid corn oil. The P/S ratio of the margarine is about 2.

This number is determined by dividing the grams of polyunsaturated fat by the grams of saturated fat listed on the Nutrition Information label. A margarine with a P/S ratio of less than 2.0 will not produce a total diet P/S ratio of 1.0 to 1.3.

The meats allowed in the diet have an average cholesterol content of 30 mg or less per ounce. This includes the use of Brown's (1976) recommendations of low and medium fat meats such as lean beef, pork, ham, Canadian bacon, lamb, veal, fish and poultry without the skin. The meat group is limited to a total intake of 7 ounces/day or 210 mg cholesterol.

NUTRITION INFORMATION PER SERVING

SERVING SIZE	14g
SERVING PER CONTAINER	32
CALORIES	100
CARBOHYDRATES	0g
FAT (100% OF CALORIES)	11g
POLYUNSATURATES*	**4g**
SATURATES*	**2g**
CHOLESTEROL* (0 mg/100g)	0mg

PREPARED FROM **PARTIALLY HARDENED SOYBEAN OIL**
AND COTTONSEED OIL, PASTEURIZED SKIMMILK, SALT, MONO
& DIGLYCERIDES; POTASSIUM SORBATE ADDED AS A PRE-
SERVATIVE, CITRIC ACID ADDED TO PROTECT FLAVOR,
ARTIFICIALLY COLORED WITH BETA CAROTENE. VITAMIN
A PALMITATE ADDED.

* Information of fat and cholesterol content is provided for indivi-
duals who, on the advice of a physician, are modifying their total
dietary intake of fat & cholesterol.

0   43000 79420

Fig. 8.   Example of an unacceptable margarine. Note that the pri-
mary ingredient is a hydrogenated vegetable oil. This
makes the product unacceptable, even though the P/S ratio
is 2.

Skim milk and foods made with skim milk may be used as desired.
If 2% milk is preferred, 8 ounces may be incorporated into the
daily meal plan or 16 ounces of 1% milk may be substituted.

The desired P/S ratio is achieved by using 2 to 3 tablespoons
of margarine or acceptable oils each day. The acceptable oils
are liquid safflower, liquid soybean, liquid corn, liquid cotton-
seed, liquid sesame seed and liquid sunflower seed oil. The use
of salad dressings, mayonnaise and stir fried vegetables is
suggested to reach the recommended amount (Figures 9 and 10).
Most brands of commercial mayonnaise have a high P/S ratio.
However, these products do contain egg yolk which will affect the
cholesterol content of the diet if used on a regular basis.
Therefore, our patients are advised to use mayonnaise sparingly.
An excessive intake of margarines or oils, i.e. >3 tablespoons/day
has been associated with the potential risk of cholelithiasis
(Sturderant, 1973). Therefore, we strongly suggest that to
achieve the desired daily P/S ratio, the saturated fat intake be
drastically reduced and the polyunsaturated intake increased up

INGREDIENTS: **Soy-
bean Oil**, Glucose Syrup,
Sugar, Water, Vinegars,
Tomato Paste, Salt, Paprika,
Dehydrated Onion and
Garlic, Natural Flavorings
and Spices, Algin Derivative
Added.

0 43000 79420

Figure 9.   Ingredients of an acceptable oil useful for
             achieving the desired P/S ratio.

INGREDIENTS: **PARTIALLY HYDROGENATED SOYBEAN OIL,** WATER,
SUGAR, CORN SYRUP, RED WINE VINEGAR, TOMATO PASTE, SALT,
LEMON JUICE CONCENTRATE, DRIED ONION AND GARLIC,
XANTHAN GUM (IMPROVES
POURABILITY), SORBIC ACID (A
PRESERVATIVE), SPICES, NAT-
URAL FLAVORING, CALCIUM
DISODIUM EDTA (A PRESERVA-
TIVE), ARTIFICIAL COLORING.

0 43000 79420

Figure 10.   Ingredients of an acceptable oil useful for
             achieving the desired P/S ratio.

to 3 tablespoons of acceptable vegetable oils.

Table 4. Prudent Polyunsaturated Fat Diet Calculations[a]

	Fat gms	Sat. FA gms	Poly FA gms	Chol mg
7 oz. lean beef (7 x 3 gms)	21	9[b]	1.2[b]	196[c]
2 c. 1% milk (2 x 2.5 gms)	5	3.1[d]	0.2[d]	14[e]
4 tsp. tub marg. (4 x 4 gms)	16	2.4[f]	4.8[f]	-
4 tsp. corn oil (4 x 5 gms)	20	2.6[g]	11.6[g]	-
Total:	62	17.1	17.8	210
Calories:	558	153.9	160.2	
% Total Calories Based on 1800 Calories	31	8.5	8.8	
Total P/S Ratio		1.0		

a – Adapted from Brown, 1978.
b – Beef fat Sat. = 43% Poly. = 6%
c – Beef chol. = 28 mg/oz.
d – Butter fat Sat. = 62% Poly. = 4%
e – Milk 1% chol. = 7 mg.
f – Tub marg. Sat.= 15%
        Poly. = 30%
g – Corn oil Sat. = 13%
        Poly. = 58%

The PPFD excludes the use of various meat items such as convenience luncheon meats, frankfurter, sausage, bacon, poultry skin, commercially prepared meats and fish, TV dinners, commercially canned soups, stews and similar products. The reasons that they are excluded are twofold. The first is due to the high saturated fat content and/or cholesterol content greater than 30 mg per ounce. The second reason is due to the lack of nutrient information regarding the total fat, saturated fat and cholesterol content of commercially prepared convenience food items. (Table 5)

Table 5 illustrates examples of the cholesterol content of selected food items. Degree of hydrogenation is also important. Hydrogenation changes the chemical structure of an oil from an

Table 5. Cholesterol Content of Selected Foods

Cholesterol/mg	Food	Amount
over 1500	Brains	3 oz. raw
500-1500	Kidneys	3 oz. cooked
200-500	Liver (beef, calf, hog, lamb)	3 oz. cooked
	Heart, beef	3 oz. cooked
	Egg yolk	1
100-200	Clams, halibut, tuna	3 oz. cooked
	Chicken, turkey (light meat)	3 oz. cooked
	Beef, pork, lobster, chicken, turkey dark meat)	3 oz. cooked
	Lamb, veal, crab	3 oz. cooked
20-50	Whole milk	8 oz.
	Cream, light	1 oz.
	Creamed cottage cheese	1/2 cup
	Processed American cheese	1 oz.
	Processed Swiss cheese	1 oz.
	Ice cream	1/2 cup
	Cheddar cheese	1 oz.
	Frankfurter	1
	Butter	1 Tbsp.
	Cake (from commercial mix)	2-1/2 oz.
	Oysters, salmon	3 oz. cooked
under 20	Half and half	1 oz.
	Low fat yoghurt	8 oz.
	Lard	1 Tbsp.
	Mayonnaise	1 Tbsp.
	Uncreamed cottage cheese	1/2 cup
	Skim milk	8 oz.

unsaturated fat to a saturated fat. If a vegetable oil is solid at room temperature or lists a partially hardened or hydrogenated oil as the first word on the list of ingredients, it should be avoided because it is now a saturated fat. All vegetable oils are not high in polyunsaturated fats. (Table 6) Most nondairy creamers and whipped toppings are made from coconut oil or palm oil which are saturated fats. Labels of products that state either "vegetable fat" or "vegetable oil" are usually one of these two oils and should be avoided.

Table 6. Fatty Acid Composition of Various Oils
            (percent distribution)[a]

Fatty Acid	Coconut Oil	Palm Oil	Olive Oil	Corn Oil	Safflower Oil
C 8:0	5.40	-	-	-	-
C 10:0	8.40	-	-	-	-
C 12:0	45.42	0.13	-	-	-
C 14:0	18.10	0.91	0.08	-	-
C 16:0	10.53	38.42	11.91	13.1	-
C 16:1	0.40	-	1.05	-	0.90
C 18:0	2.30	4.57	2.48	4.07	-
C 18:1	7.51	42.44	73.24	29.01	17.30
C 18:2	-	12.42	9.07	54.00	79.00
C 18:3	-	0.71	1.01	-	0.13
Others	1.94	0.40	1.16	-	2.67

a - Naito, H.K. (Unpublished report, determined by gas-liquid
    chromatography.)

PRACTICAL CONSIDERATIONS OF THE DIET

     Information on brand names, label reading and food preparation
may permit their use and help the palatability and variety of the
diet. It is important that the diet be a positive change, en-
couraged by physicians, dietitians and family members. By provid-
ing information on label reading, food preparation and dining out,
patients are offered a liveable diet. The food industry continues
to provide new food products which dietitians must evaluate for
the cholesterol and saturated fat content. The dietitian's pro-
fessional judgement, in many instances, is the only guideline for
evaluating the product.

     By law, ingredients are listed on the label in descending
order of the content by weight. Many items such as breads and

cereals contain small amounts of saturated fats. Such products
are allowed if the saturated fat is not listed as one of the
first five ingredients (Figures 11 and 12).

INGREDIENTS: UNBLEACHED ENRICHED
WHEAT FLOUR (FLOUR, NIACIN, REDUCED
IRON, THIAMINE MONONITRATE (VITAMIN
B_1), RIBOFLAVIN (VITAMIN B_2). CRACKED
WHEAT, WATER, 100% STONE GROUND
WHOLE WHEAT FLOUR, CORN SYRUP, UN-
SULPHURED MOLASSES, **PARTIALLY HY-
DROGENATED VEGETABLE SHORTEN-
ING** (SOYBEAN, PALM AND/OR COTTONSEED
OILS), YEAST, WHEAT GLUTEN, SALT,
HONEY, VINEGAR AND MONO- AND DIGLYC-
ERIDES (FROM HYDROGENATED VEGE-
TABLE OIL).

0   43000 79420

Fig. 11.   Example of an unacceptable grain product. Although the
           type of fat used is saturated, note that it is not the
           primary ingredient.

INGREDIENTS Enriched Flour (Flour, Niacin, Reduced
Iron, Thiamine Mononitrate, Riboflavin), Sugar,
**Vegetable Shortening (contains one or more of the
following partially hydrogenated oils: Soybean,
Cottonseed, Palm, Peanut)**, Graham Flour, High Fructose
Corn Syrup, Honey, Salt, Baking Soda, Artificial Flavor

0   43000 79420

Fig. 12.   Example of an unacceptable grain product. Note that the
           saturated fat used is listed 3rd and is a primary in-
           gredient.

     Several low cholesterol cookbooks are available that our
patients have found to be helpful. Not all cookbooks contain
accurate information but we recommend three to our patients
(Brown, 1968; American Heart Association, Inc., 1973; Jones,
(1975).

Correct food preparation encourages creativity and increases variety in the diet. Table 7 provides a substitution list for high-cholesterol containing food products. With the elimination of many convenience food items, meal preparation will become more time consuming. Soups, casseroles and baked goods must be home-made with acceptable ingredients. The amount of counseling and cooking suggestions given by the dietitian to the patient will vary depending on a patient's previous eating pattern. Suggestions include trimming the fat from all meat before cooking and removing the skin from all poultry. The meat should be weighed after cooking with the bone and the fat removed. Meats can be baked, broiled or roasted on a rack. This will keep the meat above the fat drippings. Meats can be made moist by adding tomato juice, lemon juice, wine, broth, water or an acceptable oil combined with herbs and spices. Low fat gravies are made by refrigerating the meat drippings until the fat has hardened. The hardened fat can be skimmed off and removed and the gravy may be made from the remaining natural juice which contains a negligible amount of fat. Any acceptable vegetable oil or margarine may be added to the natural juices.

Combination dishes such as soups, stews and casseroles are prepared by cooking the meat separately and removing the fat either by draining the meat or chilling it overnight.

The vegetables should be prepared in the usual manner using acceptable margarines or oils in place of other unacceptable fats. Stir frying can be tried for variety. Recipes using egg whites, cholesterol free egg substitutes and skim milk products should be encouraged.

Dining out in restaurants is an important part of the American lifestyle. This is an area that is frequently overlooked and the dietitian needs to give the patients practical suggestions to help them maintain their diet. Most restaurants and cafeterias offer enough variety of foods so that a prescribed meal pattern can be selected. Restaurants that specialize in one product, i.e. omelettes or ice cream are not the wisest choice. Waiters and waitresses are usually helpful in explaining how an item is prepared. Many times they can have the food item prepared without the butter or cream sauce. Counseling patients regarding food choices in a restaurant is a difficult aspect of dietary management. In many instances the frequency of meals eaten in restaurants will need to be reduced but not omitted totally from the dietary proto-col. Although dining out tends to violate the dietary recommen-dations of the PPFD, the suggestions offered in Table 8 list food choices which can be substituted on limited occasions. Cooperation of the restaurant industry regarding food preparation is not only needed but necessary in maximizing the therapeutic effectiveness of diets.

Table 7. Recipe Substitutions for Cholesterol Containing Foods[a,b]

Foods High in Cholesterol and/or Saturated Fat	Recipe Substitutions
Marbled red meats, bacon, sausage, hot dog, luncheon	Lean red meats, pork, ham, Canadian Bacon, poultry, fish and peanut butter* Limit use to 7 oz./day. *Old fashioned or homemade may be used as desired.
Egg yolk Limit to 2/week, including those used in cooking	Cholesterol free egg substitutes and egg whites.
Whole milk	Skim milk, evaporated skim milk, reconstituted non-fat dry milk. Limited amounts of 1% or 2% milk.
Cream, half and half, non-dairy creamers	Cream substitutes made from acceptable oils, evaporated skim milk or non-fat dry milk mixed to double or triple strength.
Cheese	Low fat cheese containing 5% milk fat or less. May use 1 oz. of regular cheese in place of 2 oz. of meat, fish or poultry.
Sour cream	8 oz. low fat plain yoghurt + 1 teaspoon cornstarch.
Cream cheese	Low fat cottage cheese blended with low fat yoghurt or skim milk to desired consistency.
If a recipe calls for:	Use instead:
1 egg yolk	1 egg white or 1 egg white + 1 teaspoon acceptable oil
1 whole egg	2 egg whites or 1 egg white + 1 teaspoon acceptable oil, or 1/4 cup cholesterol free egg substitutes.

Table 7.   (Cont.)

2 whole eggs	3 egg whites or 2 egg whites + 2 teaspoons acceptable oil, or 1/2 cup cholesterol free egg substitutes
Evaporated milk	Evaporated skim milk
1 oz. baking chocolate	3 tablespoons unsweetened cocoa powder + 1 tablespoon acceptable fat
If a recipe calls for:	Use instead:
Butter or shortening	Safflower, sunflower, corn cotton-seed, soybean and sesame seed oils or acceptable margarines
Whipped toppings	Evaporated skim milk or non-fat dry skim milk powder
1 oz. bacon (2 strips)	1 oz. lean Canadian bacon or boiled ham; or 2 tablespoons of imitation bacon bits; or 2 strips of imitation bacon strips.

a, b - Adapted from: Cleveland Clinic Foundation Diet Manual, 1979.   American Heart Association Cookbook, 1973.

Table 8.   Restaurant Suggestions

Appetizers: Consomme or bouillon, fruit cup, vegetable or fruit
juice, seafood cocktail with lemon juice or acceptable sauce.

Entrees: Lean meat, poultry or fish baked or broiled without fat
or gravy. Remove poultry skin and trim fat. Limit the amount
and kind of meat according to the diet plan.

Vegetables: Any vegetable prepared without fat. Avoid all
creamed vegetables and those containing butter, cheese or sour
cream.

Salads: Vegetable, fruit and gelatin salads with dressings or
mayonnaise allowed in the diet. Avoid those containing coconut,
cream or cheese.

Breads: Sliced bread, hard rolls, English Muffins, bread sticks,
plain melba toast or crackers. Avoid rich dinner rolls, biscuits,
cornbread, popovers, muffins, pancakes, waffles and French toast.

Sandwiches: Sliced chicken, turkey, veal, lean beef and ham.
Chicken, turkey or tuna salad without chopped egg. (Ask that the
butter or margarine be omitted).

Desserts: Fresh or canned fruit, gelatin, angel food cake, fruit
ice.

Beverage: Coffee, tea, decaffeinated coffee, fruit or vegetable
juices, carbonated beverages, skim milk.

MODIFICATIONS OF THE PRUDENT POLYUNSATURATED FAT DIET

If reduction to ideal body weight is necessary, the diet can
be divided into exchange groups. At The Cleveland Clinic Founda-
tion, the current PPFD has not been developed in this manner.
However, for those individuals who are above their ideal body
weight and/or have elevated triglycerides, a modified version of
the American Diabetes Association's 'Exchange Lists For Meal
Planning' (1976) which incorporates the parameters of the PPFD is
used. If the patient is at his or her ideal body weight, a
caloric restriction is not used, but rather the patient is coun-
seled on guidelines for maintaining his/her weight. For those
patients with elevated triglycerides and at their ideal body
weight, a diet was designed restricting the use of concentrated
carbohydrates, i.e. sugar, honey, jams, jellies, soda pop and al-
cohol (Department of Nutritional Services, 1977a). This is used
in conjunction with the PPFD.

If a patient has hypertension, a 3 to 4 gm sodium restriction
is generally used. This level of restriction eliminates the use
of smoked and cured meats and fish, sauerkraut, pickles, olives
and condiments such as ketchup, mustard and steak sauce, to list a
few examples. (Department of Nutritional Services, 1977b).
Commercially prepared dinners contain a high fat content as well
as excessive levels of sodium.

In counseling pregnant or lactating women on the prudent
diet, attention must be given to their increased nutrient and
caloric needs. Vegetarians, depending on the degree of dietary
restriction, will need information on foods available and main-
taining an adequate nutrient intake. If indicated, the dietitian
must educate the vegetarian on how to combine foods to achieve a
protein intake of high biological value.

Children's diets need special consideration. The Pediatric
Lipid Clinic at the Cleveland Clinic Foundation follows children
from family members who are at high risk for premature CHD. This
clinic handles the children's diets separately from the adult
population. Because of space limitations, this topic will not be
covered here.

To ensure any guarantee of patient adherence to the dietary
protocol, dietary follow up with a dietitian is necessary and
critical. This phase is usually the weakest link of most dietary
programs. The initial diet instruction cannot cover all aspects
of the diet. Questions will arise when the individual attempts to
incorporate, at home, the diet into his or her lifestyle. Also,
additional dietary counseling can be used as a supportive mechan-
ism in optimizing patient adherence.

It is generally recognized that ongoing dietary follow up
protocols increase patient adherence and success with changes in
lifestyle. At the present time, diet follow up is patient initiated
at the Cleveland Clinic Foundation. Being a tertiary care center,
a large percentage of our patients are from out of town. At the
time of the dietary instruction, the patients are encouraged to
write or call if questions arise. We realize that this is not an
adequate system. Although the Cleveland Clinic Foundation lacks
rigid structure and consistency in the follow-up program as
carried out by the "high risk" cardiology patients, we feel that
the dietary program has been effective. Preliminary study (see
discussion below) indicates that patient adherence to the PPFD
appears to be high.

PATIENT ADHERENCE

A retrospective study was done on 49 males and 2 females
ranging in age from 34-66 years, returning to The Cleveland
Clinic Foundation for their six week post-operative check up
during January and February, 1979. All had attended the Prudent
Polyunsaturated Fat Diet class prior to hospital discharge.
During the post-operative check up, a diet survey sheet was
distributed to the patients. Questions included a 24-hour recall
designed to assess dietary adherence to the PPFD. Eighty-five
percent admitted that the diet was a change from their previous
eating habits. Sixty-nine percent stated they do not have trouble
following the diet and 65% did not find the diet too restrictive.
It was reported by 81% that their meat consumption was seven
ounces or less per day. Sixty-two percent said they could not
reduce their meat consumption below the seven ounce allowance.
Twenty percent avoided egg yolks in their diets completely and
69% ate one to two egg yolks per week. Fifty-four percent reported
using skim milk as a beverage or in cooking and 35% indicated
using 2% milk. Fifty-eight percent used the recommended amount
of polyunsaturated oils and margarines per day.

The results of the study indicate that the Cleveland Clinic's
Prudent Diet has practical applications to the hypercholesterolemic
individual in the American population. The demands of the diet
are not so restrictive that they cannot be incorporated into a
given lifestyle. It appears that the transition to the PPFD is
not particularly difficult. This is advantageous to ensure high
adherence to the dietary protocol. A therapeutic diet should be
developed in a format that meets the demands of the individual's
lifestyle or better yet, that of a given population. It is up to
the professional staff to provide the educational tools and
support mechanisms needed to alter these lifestyle changes.
Ultimately, the success of the PPFD is judged by the lowering of

the individual's blood lipid (i.e. cholesterol) levels, and our
follow up studies indicate that with good dietary adherence the
blood lipid levels of most individuals do decrease and even
"normalize."

SUMMARY

     The vast number of deaths resulting from atherosclerosis
clearly identify CHD as a leading public health problem. The
etiology of CHD is complex and multifaceted. The process of
atherosclerosis can be manipulated by controlling certain nutri-
tional risk factors. Three decades after the Framingham Study,
The Dietary Goals of the United States (Select Committee on
Nutrition and Human Needs, 1977), identify the need for increasing
the American population's awareness regarding the usefulness of
modifying the nutritional risk factors for CHD. Altering the
current American food habits to incorporate a prudent intake of
cholesterol and saturated fats, to limit total fat consumption and
to achieve and maintain ideal body weight by controlling the
quantity and quality of total calories is an important concept to
promote. A prudent diet regimen can be used for screened
individuals with atherosclerotic heart disease. The decline in
the number of deaths from cardiovascular disease is due to a
combination of many factors, one being more effective education of
the American population in controlling the primary and secondary
risk factors. It is the responsibility of physicians, dietitians,
nurses, public health officials and allied health scientists to
reinforce lifestyle changes by providing the educational tools to
those individuals already at high risk as well as to the general
population. We have discussed the rationale of the Cleveland
Clinic Foundation's PPFD, a step-by-step instruction on means of
developing a hypocholesterolemic therapeutic diet and considera-
tions needed to enhance patient adherence for a successful dietary
program.

     The following are samples of menus designed to meet the
parameters of the Prudent Polyunsaturated Fat Diet discussed in
the above section. The menus were written for the reference 50
year old male, 175 cm in height, at his ideal body weight of 70 kg
with a caloric requirement of 2000 kilocalories per day. The Case
Western Reserve University Nutrient Data Bank analyzed the menus
for nutrient adequacy, cholesterol content, P/S ratio, fiber
content and the percent of total calories derived from carbohy-
drate, protein and fat.

DAY 1

	Serving Size

Breakfast

Apricot Nectar	6 oz.
Farina	1 cup
Low cholesterol egg substitute	1/2 cup
Whole wheat toast	2 slices
Margarine	3 tsp.
Skim milk	8 oz.

Lunch

Corned beef sandwich (on rye bread)	1 (with 3 oz. meat)
Three bean salad	1 cup
Apple	1 large

Dinner

Salmon steak	4 oz.
Herb rice	1 cup
Beets	1/2 cup
Tossed salad	1 cup
Italian dressing	3 tsp.
Whole wheat bread	2 slices
Margarine	2 tsp.
Angel food cake	1-1/2" slice
Skim milk	8 oz.

Evening Snack

Old fashioned peanut butter	2 tbsp.
Crackers	8

DAY 2

Breakfast

Grapefruit	1/2
Raisin bran	1 cup
*Pancakes (*homemade with acceptable ingredients)	3
Margarine	3 tsp.
Skim milk	8 oz.

Lunch

Syrian sandwich	1 (with 3 oz. meat)
Celery sticks	
Orange	1 medium
Skim milk	8 oz.

Dinner

Pork chop	4 oz.
*Bread dressing (*homemade with acceptable ingredients)	1 cup
Wax beans	1 cup
Pan roll	2
Margarine	3 tsp.
**Bananas Flambe	

DAY 3

Breakfast

Cranapple juice	6 oz.
*Fried egg (*made with margarine)	1
English muffin	1
Margarine	3 tsp.

Lunch

Chicken salad sandwich (on whole wheat bread)	1 (with 2 oz. meat)
Mayonnaise	2 tsp.
Carrot sticks	
Apple	1 large
Skim milk	8 oz.

Dinner

Roast beef	5 oz.
Mashed potatoes	1 cup
Corn	3/4 cup
Whole wheat bread	2 slices
Margarine	3 tsp.
**Pink Lemonade pie	1/8 pie
Skim milk	8 oz.

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DAY 4

	Serving Size
Breakfast	
Orange juice	6 oz.
Farina	1 cup
Low cholesterol egg substitute	1/2 cup
Whole wheat toast	2 slices
Margarine	3 tsp.
Skim milk	8 oz.
Lunch	
Onion soup	1 cup
Ham sandwich on roll	1 (with 2 oz. meat)
Sliced tomato salad	
Mayonnaise	2 tsp.
Pear	1 medium
Skim milk	8 oz.
Dinner	
Lamb chop	5 oz.
Rice Pilaf	1 cup
Peas	3/4 cup
Mixed greens	1 cup
Italian dressing	2 tsp.
Whole wheat bread	2 slices
Margarine	2 tsp.

DAY 5

Breakfast	
Pineapple juice	6 oz.
*Omelet (*made with low cholesterol egg substitute)	1/2 cup
Rye toast	2 slices
Margarine	3 tsp.
Skim milk	8 oz.
Lunch	
Cottage cheese fresh fruit plate	1/2 cup (low fat cheese)
Melba toast	8
**Whole wheat cupcake	2

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Serving Size

Dinner

Veal Italian style	6 oz.
Noodles	1 cup
Zucchini	1 cup
Fresh spinach salad	1 cup
French dressing	3 tsp.
Kaiser roll	2
Margarine	2 tsp.
Fresh fruit cup	1 cup

DAY 6

Breakfast

Orange juice	6 oz.
Poached egg	1
Canadian bacon	2 oz.
Whole wheat toast	2 slices
Margarine	3 tsp.

Lunch

Roast beef sandwich (on whole wheat bread)	1 (with 2 oz. meat)
Pineapple chunks	3/4 cup
**Cinnamon nuts	1 cup
Skim milk	8 oz.

Dinner

Apricot nectar	6 oz.
Baked fillet of flounder	3 oz.
*Delmonico potatoes (*made with acceptable ingredients)	1 cup
Green beans	3/4 cup
Coleslaw	3/4 cup
Whole wheat bread	2 tsp.
Margarine	3 tsp.

Evening Snack

*Banana/Strawberry milkshake (*made with
    skim milk)

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DAY 7

Serving Size

Breakfast

Banana	1 medium
Special K	1 cup
*French toast (*made with low cholesterol	2
egg substitute and wheat bread	
Margarine	3 tsp.
Skim milk	8 oz.

Lunch

Tomato juice	6 oz.
Baked perch	4 oz.
Herb rice	1 cup
Parslied carrots	1/2 cup
Rye roll	2
Margarine	2 tsp.
Skim milk	8 oz.

Dinner

Spaghetti with meat sauce	2 cups (with 3 oz. meat)
Tossed salad	1 cup
Italian dressing	3 tsp.
Italian bread	2 (1" slices)
Margarine	1 tsp.
Melon	1 cup

DAY 8

Breakfast

Orange juice	6 oz.
Life cereal	1 cup
Low cholesterol egg substitute	1/4 cup
English cup	1
Margarine	3 tsp.
Skim milk	8 oz.

Lunch

Hamburger on bun	1 (3 oz. pattie)
Lettuce and tomato slices	
Grapes	20

	Serving Size

Iced tea

Dinner

Ham steak	4 oz.
Sweet potatoes	1 cup
Cauliflower	3/4 cup
*Biscuit (*homemade with acceptable ingredients)	3
Lettuce wedge	
French dressing	2 tsp.
Margarine	3 tsp.
Skim milk	8 oz.

DAY 9

Breakfast

Grapefruit sections	1/2 cup
Oatmeal with raisins	1 cup (with 1/4 cup raisins)
Bagel	1
Margarine	2 tsp.
Skim milk	8 oz.

Lunch

Tuna salad sandwich (on whole wheat bread)	1 (2 oz. tuna)
Mayonnaise	2 tsp.
Fresh fruit cup	1/2 cup
Angel food cake	1-1/2" slice
Skim milk	8 oz.

Dinner

Roast pork	5 oz.
Mashed potatoes	1 cup
Broccoli	1/2 cup
Marinated beet and onion salad	3/4 cup
Whole wheat bread	1 slice
Margarine	3 tsp.
*Pudding (*made with skim milk)	1/2 cup

Evening Snack

Popcorn, popped	2 cups
Iced tea	2 cups

DAY 10

	Serving Size
Breakfast	
Orange juice	6 oz.
40% Bran Flakes	1 cup
Low cholesterol egg substitute	1/4 cup
Whole wheat toast	2 slices
Margarine	3 tsp.
Skim milk	1 cup
Lunch	
Roast turkey sandwich (on whole wheat bread)	1 (3 oz. meat)
Mayonnaise	2 tsp.
Carrot and celery sticks	
Apple	1
Dinner	
Broiled steak	4 oz.
Baked potato	1 large (7 oz.)
Asparagus	1/2 cup
Tossed salad	1 cup
Oil and vinegar dressing	2 tsp.
French bread	1" slice
Margarine	3 tsp.
Strawberries	1 cup

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EFFECTS OF HYPOPROTEINEMIA ON SERUM LIPOPROTEIN COMPOSITION OF

DOGS AND RATS

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INTRODUCTION

The significance of protein composition of serum in relation to
the pattern of serum lipids and lipoproteins has not been clearly
elucidated previously. Clinical investigations of protein depleted
human beings have included extensive studies of patients with nephro-
sis and with malnutrition. In hypoproteinemic patients with nephro-
sis the increased concentration of serum cholesterol and triglyceride
is carried in the –S 20-40 and –S40-70 low density (LDL) and –S
70-400 very low density (VLDL) and chylomicron fractions. The con-
centration of the –S 0-10 high density (HDL) fraction is usually not
increased and may be decreased (Lewis, L.A., 1980). Similarly in
rats with the experimental nephrotic syndrome, the increased concen-
tration of serum cholesterol is chiefly in the LDL fraction (Lewis
and Heyman, 1954). In both species the hypoproteinemia accompanies
renal damage with proteinuria. These lipid and lipoprotein changes
are in contrast with those reported in individuals with low serum
protein levels due to malnutrition. Coward and Whitehead (1972)
found in children with protein-calorie malnutrition a decrease in
serum total cholesterol, triglyceride and β-lipoprotein concentra-
tions. To determine what effect hypoproteinemia not due to renal
disease exerts on the serum lipoprotein pattern dogs with normal
renal function were depleted of protein reserves by a combination of
plasmapheresis and feeding of a protein-free diet, and rats depleted
by feeding of a protein-free diet. The serum lipoproteins and
cholesterol were studied during the period of depletion and of re-
covery, when a nutritionally adequate diet was reinstituted.

METHODS

Dog Study

     Normal adult mongrel dogs weighing between 10 and 15 kg were
studied. The animals were fed a diet of dog chow* and cooked beef.
After control blood studies were obtained after an 18-hour fast, the
dogs were placed on a protein-free diet, described by Allison and
Anderson (1945). One hundred fifty to 200 ml of blood, depending on
the size of the dog, was removed, the cells were repeatedly washed
and then returned to the animals. This procedure was repeated every
other day for three bleedings. Blood samples were collected weekly
for biochemical analyses. After four weeks on the protein-free diet
the dogs were returned to the complete ration on which they were
before protein-depletion and studies continued for nine weeks.

Rat Study

     Pathological changes, described over many years, that occur in
young children during protein deprivation vary greatly from those
that occur in the adult with protein restriction. To learn more
about the changes induced in the lipoprotein pattern during a period
of very slow growth and of much more rapid development, two groups
of rats were studied. The first was a group of old males weighing
between 500 and 600 g at the beginning of the study, the second group
comprised young, sexually mature, but still rapidly growing, male
rats. The experimental procedures used for each group follow:

    Large old rats. Seven adult male Sprague-Dawley rats weighing
between 500 and 575 g, which had been maintained on chow diet, were
bled from the tail after an 18 hour fast, for serum cholesterol and
protein determination. They were then placed on the above protein-
free diet (Allison and Anderson, 1945) with supplementary vitamins
being given twice weekly. The rats had free access to food at all
times except the 18 hours before collection of blood samples. Small
samples of blood were taken after the rats had received the special
diet for four weeks and eight weeks, and after ten weeks they were
sacrificed and blood collected for analyses. (See below, Analytical
Procedures C).

    Young adult rats. (a) Effects of protein free diet on serum
lipoprotein and lipids. A group of 12 young, male rats, average
weight 285 g, was studied. After initial blood analyses when they
were on the chow diet**,eight of the rats were placed on the protein-

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*   Ken-L-Ration, Quaker Oats Co., Chicago, Illinois 60654
**   Wayne rat chow, Allied Mills, Special Feed Department, N.
    Wacker Drive, Chicago, Illinois 60606

Table 1. Experimental Diet for Rats (Diet I).

High Fat Diet	gm	% of Calories
egg yolk	10	P = 21.3
		F = 50.4
soya oil	21	C = 28.3
nonfat milk solid	65	
salt mixture (Osborn Mendel)	3	
Vitamins NBC – Vitamin diet fortification mix contains 100 mg choline/100 gm	1	

Table 2. Solutions for Parenteral Nutrition for Rats

Base Solution	Solution A Fat-free	Solution B Fat-Supplemented
8.5% Freamine	500 ml	500 ml
50% Dextrose	500 ml	500 ml
Sterile H_2O	109	–
10 Intralipid	–	309
Additives		
10% Ca glucomate	10 (4.6 mEq)	10
50% $MgSO_4$	0.2 (0.8 mEq)	0.2
14.9% KCl	35 (70 mEq)	35
23.4% NaCl	8 (32 mEq)	8
16.4% Na acetate	8 (16 mEq)	8
MVI	5	5

free diet and four were continued on the chow. After six and ten
weeks, blood studies were made.

     (b) Effects of protein-adequate diet after protein depriva-
tion on serum lipoprotein and lipids. A second group of 20 young
rats, average weight 298 g, were studied. The plan of study was
the same as described above except that the rats were returned to
one of two nutritionally complete diets after protein depletion.
Diet I was a high energy diet, high in fat containing 50.4% fat,
21.3% protein and 28.3% carbohydrate (Table I). Diet II was the
standard rat chow*. Six weeks after the rats were returned to
nutritionally adequate diet they were sacrificed and blood lipids,
lipoproteins and proteins determined.

     (c) Effects of adequate amino acid nutrient given parenterally
after protein deprivation on serum lipoprotein and lipids of rats.
A third group of 24 young mature rats, average weight 260 g, was
similarly depleted of protein by dietary means for six weeks. At
that time eight were sacrificed and the remaining received intra-
venous infusions of fluid to provide complete nutrition paren-
terally. Eight received solution A, basic solution which was fat
free, eight received solution B, which contained additional intra-
lipid (Table 2). After seven days of parenteral protein repletion
the rats were sacrificed. In addition to serum analyses, liver
samples were studied by electron microscopy.

Analytical Procedures

     Lipoproteins were determined ultracentrifugally at a density
of 1.063 (Gofman, 1945) and/or of 1.21 (Lewis, 1952), or by electro-
phoresis on paper (Lees, 1960), acrylamide gel electrophoresis
(Naito, et al., 1973) or on agarose gel (Pfizer System, 1974).
Cholesterol was measured by the method of Abell et al. (1952) or by
the autoanalyzer-II technique, by the protocol of the Lipid Research
Clinics (1974); total protein by the biuret method (Sols, 1947).
Starch gel electrophoresis was performed by the thin-layer modifi-
cation (Lewis, 1966) of the method of Smithies (1962), using a dis-
continuous system of buffers (Poulek, 1957). Gels were stained for
protein or lipid using amidoschwartz 10B or oil red-O. Electro-
phoretic studies of serum proteins were made on paper by the method
of Durrum (1958) using barbiturate buffer, pH 8.6, or acetate by
the microzone technique (1973). The livers of the rats were
studied by light microscopy and some also by electron microscopy
(EM). Details of the EM procedure have been summarized by Blanchard
et al. (1979).

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*Wayne rat chow, Allied Mills, Special Feed Department, N. Wacker
 Drive, Chicago, Illinois 60606

RESULTS

The lipids in the serum or plasma are transported bound with protein in the form of lipoproteins. The lipoproteins have been separated and characterized chiefly by two methods, ultracentrifugation and electrophoresis. By ultracentrifugation of serum after increasing the density by addition of salts to an appropriate level, the lipoproteins are floated to the top of the tube in a way similar to the separation of cream in a separator. The lipoprotein concentrate is removed and studied in the analytical ultracentrifuge, where lipoproteins of different densities determined by the proportion of lipid to protein in the molecule are demonstrated to have different flotation rates. Most frequently used densities for lipoprotein studies are d 1.063 (Gofman, 1949) at which lipoproteins of lower densities are floated (VLDL and LDL) and d 1.21 (Lewis, 1952) at which both lower density and high-density lipoproteins are concentrated. Table 3 represents the comparison between the terminology of fractions resolved at d 1.063 and d 1.21, and also includes the electrophoretic characteristics of the fractions.

Dog Study

Normal Dog Lipoprotein Pattern. Normal mongrel dog's serum has a cholesterol concentration which varies greatly from animal to animal, but irrespective of the cholesterol level the lipoproteins normally are concentrated predominantly in the high density, α-lipoprotein fraction with flotation rate between -S 0 and 10. The -S 0-10 fraction which is present in the amount of 400 to 1000 mg per dl is usually resolved in the ultracentrifuge as a double peak with maximum deflection at -S 4 and -S 7. The -S 4 is the more concentrated. There is often a small amount of material with flotation rate between -S 10 and 20; this fraction may be referred to as intermediate density lipoprotein (IDL). The lower density lipoproteins, -S 20-40, may be resolved as a single or double peak with maximum deflection at -S 23, and -S 28. This fraction when studied by paper electrophoresis migrates in the β-globulin area. The concentration of this fraction is normally less than 100 mg/dl. The fractions with flotation rate between -S 20 and 40 at d 1.21, are resolved in the S_f 0-12 class at a density of 1.063.

When studied by starch-gel electrophoresis, dog lipoproteins may show four clearly resolved fractions with relatively fast mobility in the range of the α-globulins, and one or two fractions with very slow mobility. The most concentrated and deeply stained fraction with amido schwartz protein stain migrates most rapidly and has a mobility approximately 0.90 X that of dog albumin in starch gel when tris-borate buffer is used.

Table 3. Nomenclature of Lipoproteins: Electrophoretic vs.
          Ultracentrifugation Techniques

Method of Study	Name of Fraction
Electrophoresis	α-lipoprotein - lipoprotein with electrophoretic mobility of α_1-globulin. They may be resolved as single or multiple bands depending on species from which serum was derived and on type of support media.
Ultracentrifugation	HDL, high density lipoproteins of d 1.063-1.21. HDL$_1$ subclass of HDL, d 1.063 - 1.080 HDL$_2$ subclass of HDL, d 1.080 - 1.125 HDL$_3$ subclass of HDL, d 1.125 - 1.21 HDL fraction has flotation rate at d 1.21 of -S 0-10. HDL does not float at d 1.063
Electrophoresis	Pre β-lipoprotein - lipoproteins with electrophoretic mobility of α_2-globulins, when separated on agarose, paper or starch powder. Pre βs may be resolved as a single or as multiple bands depending on nature of serum being studied.
Ultracentrifugation	VLDL, very low density lipoproteins, d<1.006 g/ml). At d 1.21, fraction with flotation -S70-400 includes part, but not all of VLDL. At d 1.063, S_f20-400 is VLDL. The more rapidly floating fractions are less dense, have larger molecules.
Electrophoresis	β-lipoprotein, lipoprotein with electrophoretic mobility of β-globulin. It may be resolved as single or double band, depending on support medium and buffer system used.
Ultracentrifugation	LDL, low density lipoprotein d 1.006 - 1.063 g/ml LDL$_1$ subclass of LDL, d 1.006 - 1.019 g/ml LDL$_2$ subclass of LDL d 1.019 - 1.063 g/ml LDL has flotation rate at d 1.21 of -S 25-70 or at d 1.063 S_f0-20. May be divided into subfractions, for example, -S 40-70 or S_f12-20 and -S 25-40 or S_f0-12.

Table 3.   Nomenclature of Lipoproteins: Electrophoretic vs. Ultracentrifugation Techniques (Continued)

General Comments

    In summary, low-density lipoproteins when studied by electrophoresis have electrophoretic mobility of β-globulin and immunologic properties of β-lipoprotein. High-density lipoproteins have electrophoretic mobility of α_1-globulin and immunologic characteristics of α-lipoproteins. Very-low-density lipoproteins have electrophoretic mobility of α_2-globulin.

Table 4.   Lipoprotein Changes During Protein Depletion of Dog

DOG G

Day of Study	T.P.[a] g/dl	Alb.[b]	Chol.[c]	-S	20-40	10-20	5-10	0-5
						mg/dl		
1	6.4	-	210		75	10	210	600
2	6.38	3.7	222					
150 ml blood plasmapheresis protein-free diet started.								
	5.2		297		182	157	225	400
9	200 ml							
11	150 ml							
16	4.5	-	361		300	177	258	600
23	4.3	1.60	397		250	208	300	575
30	4.2	1.39	484		325	242	218	678
Complete diet resumed.								
37	5.1	1.58	326		236	232	-552-	
44	5.5	2.63			135	164	295	450
69	6.0	3.91	318		189	166	400	782
75	5.3	3.84	313		141	54	345	465
83	5.8	3.70	287		101	90	402	402
90	5.8	3.87						
97	6.1	3.39	270		124	71	276	500

a - T.P. = Total Protein; b - Alb. = Albumin; c - Chol = Total Cholesterol.

Effect of Protein Depletion on Serum Lipoproteins of Dogs.
During the period of protein depletion, the dogs lost between 4 and
9 percent of their initial body weight. Their serum total choles-
terol concentration usually increased, in some cases by as much as
200 percent (Fig. 1). There was no close correlation between the
degree of hypoalbuminemia and the cholesterol increment. The serum
triglyceride level was slightly but not significantly increased
from a pretreatment level of 62 ± 18 mg/dl to a level of 85 ± 23
mg/dl (P >0.05) at the time of most marked hypoproteinemia. The
lipoprotein pattern was greatly modified. The increased cholesterol
was carried in all normally occurring lipoprotein fractions (-S 0-40),
but a relatively much greater proportion occurred in -S 10-20 (IDL)
and -S 20-40 (LDL) components than in the normal (Table 4). The
-S 5-10 (HDL) fraction also increased relatively more than the
-S 0-5 (HDL) fraction. There was no increase in -S 40-70 (LDL)
fraction or less dense, -S>70, lipoproteins. In the starch-gel
electrophoretic studies, increased concentration of slower migrating
fractions in the slow α and β range was observed. The slow α com-
ponent in some cases was greatly augmented.

In one dog the serum cholesterol concentration decreased to
the normal level, while the animal was still receiving the protein-
free ration. The lipoprotein pattern showed a lower -S 0-5 con-
centration, and higher -S 5-10 and -S 10-20 (IDL) fraction than when
the animal was eating a complete ration, but there was no increment
in -S 20-40 lipoproteins. The dog had a long, lean body build, and
lost only 4 percent body weight while on the protein-free diet.

Effect of Protein Repletion on Serum Lipoproteins of Dogs
after Protein Deprivation. When the animals were returned to a
complete ration the lipoprotein pattern gradually returned to the
original normal pattern. The speed of return to normal varied, but
was usually complete in six weeks. Serum total protein and protein
electrophoretic pattern became normal at about the same time as the
lipoprotein (Table 4).

Rat Study

Serum lipoprotein pattern of healthy, mature rats. The serum
lipids of rats as in most mammalian species are transported in
lipoprotein fractions with the electrophoretic mobility of α_1 and
β-globulins and flotation rate at d 1.21 between -S 0 and -S 40.
Unlike the dog ultracentrifugal lipoprotein pattern the HDL (α-lipo-
proteins) have a more diffuse contour. Both species unlike most
adult human beings have a greater proportion of lipids transported
in HDL than LDL. In the healthy rat usually less than 15 percent
of the total serum lipoprotein is present as LDL.
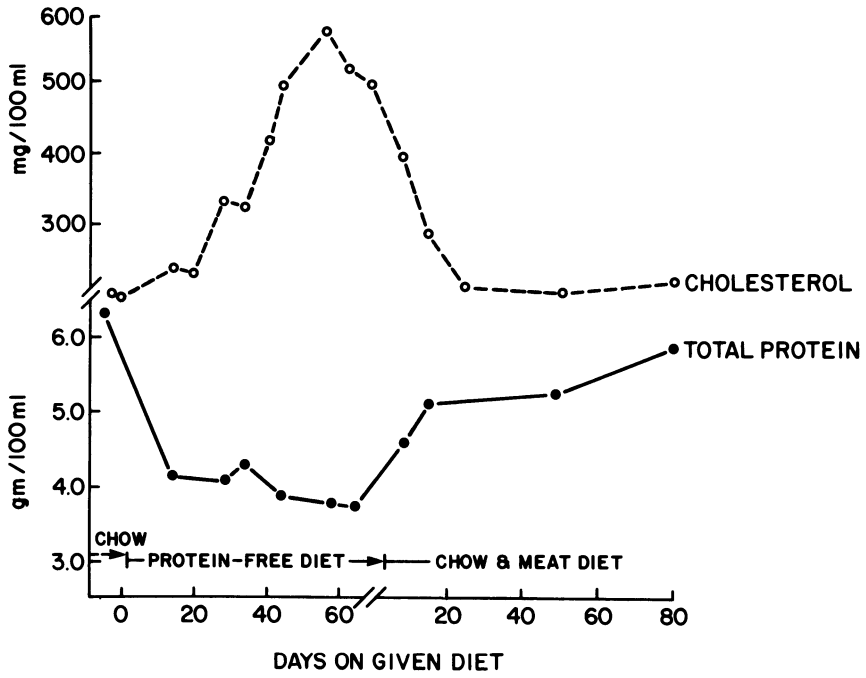
Fig. 1.   Serum total cholesterol and total protein changes during
canine protein depletion and repletion.

Effect of Protein Deprivation on Lipoprotein Distribution of Large, Old Rats. After four weeks of protein deprivation, the serum cholesterol levels of four of the rats increased slightly (15 percent) while three did not change from the pretreatment level. During this period, six of the seven rats each lost between 15 and 20 percent body weight, while that of the seventh was stable. The serum total protein concentration did not change. After ten weeks on the diet, the serum cholesterol levels decreased 11 percent from the initial level of 85.4 + 7.6 to 76.1 + 9.8 P >0.05, and serum triglyceride increased 93 percent from 72.0 + 7.0 to 138.9 + 4.5 mg/dl P <0.001. The animals lost an average of 34 percent of body weight. The lipoprotein patterns of six rats were markedly changed, 6/6 showing appreciably increased concentration -S 70-400 VLDL, and 4/6 also showing increase in -S 40-70 LDL; 0/6 showed altered HDL-lipoprotein. Normal rats of similar weight when fed chow diet showed insignificant amounts of -S 70-400 VLDL and -S 40-70 LDL lipoproteins, all of the lipoproteins being in -S 20-40 LDL and -S 0-20, HDL, fractions. The total serum protein concentration of all animals on protein-free diets decreased, from a mean of 6.21 + 0.19 to 4.13 + 0.33 g/dl (P <0.001) and the albumin from 4.60 + 0.33 to 2.93 + 0.45 g/dl (P <0.001). The livers of all the old, protein depleted animals were visibly fatty, and microscopic examination showed fatty infiltration. Livers of normal healthy rats on a nutritionally adequate diet showed no lipid by gross or microscopic examination.

Effects of Protein Deprivation on Young Rats. After six weeks on the protein-free diet, the body weight of the rats had not changed, (average pre-diet weight 286, six week weight 287 g). During this period the rats on chow diet had gained 34 percent in weight. The serum cholesterol level of the protein deficient group increased from 73 to 101 mg/dl, while that of the chow fed animals was not significantly changed being 76 and 82 mg/dl, respectively. The serum total protein level of the chow group increased slightly but not significantly from 6.4 to 6.7 g/dl while that of the protein deficient group decreased significantly from 6.2 + 0.10 to 5.3 + 0.12 g/dl (P <0.001) during the ten weeks. The albumin decreased from 3.4 + 0.2 to 2.3 + 0.5 g/dl and there was an increase in the concentration of proteins of α_1-globulin mobility from 0.9 + 0.15 to 1.4 + 0.18 g/dl (P <0.02). The concentration of serum albumin and α_1-globulin of the chow fed animals had not changed significantly from the initial levels.

The lipoprotein pattern of normal rat serum when studied by acrylamide gel electrophoresis showed multiple lipid stained bands of α-globulin mobility and smaller amounts of lipid stained material with mobility between that of α and β-lipoprotein, and a small well resolved fraction with mobility of β-lipoprotein. The most rapidly migrating part of the α-lipoprotein was present in

greatest concentration. After six weeks on the protein deficient
diet the relative concentration of the fast-migrating α-lipopro-
tein fraction decreased while that of slower migrating α-lipopro-
tein, and the fraction of intermediate mobility increased. The
concentration of the β-lipoprotein was not changed. After ten
weeks of protein depletion the percent of α-lipoprotein of fast
mobility (α_1-globulin) by the acrylamide gel technique had de-
creased being 34 percent (range 28-44%) of the total α-lipoprotein
in the depleted animal and 56 percent (range 50-68%) in the chow
fed. The concentration of fractions of slow electrophoretic
mobility (i.e., β and pre-β lipoprotein) was greater in the de-
pleted than in the chow-fed animals.

Protein Depleted Young Rats When Returned to a Nutritionally and
Protein Adequate Diet

The rats in the second study of protein deprivation of younger
animals showed the same type and degree of change as group 2
described in the previous section which included a significant
increase in the concentration of serum cholesterol after ten weeks
of protein deprivation (Table 5). During the first week on the
diet the animals continued to gain weight, but after ten weeks on
the PFD had lost, so that they were near their weight when protein
depletion was started. There was no change in triglyceride con-
centration. The rats that were returned to a chow diet had re-
gained after six weeks the weight lost during the last weeks of
protein deprivation while those on the high fat diet did not gain
quite as rapidly. The serum cholesterol level of both groups
during protein repletion decreased, but neither fell to the pre-
depletion level of 74 mg/dl. Normal, continuously chow fed rats
of similar age showed serum cholesterol concentrations of 77 $\pm$ 0.8
and triglyceride of 77 $\pm$ 3 mg/dl. The serum lipoprotein patterns
of both groups of rats after six weeks of protein repletion were
less abnormal than at the period of maximum protein depletion.
There was considerable variation between patterns of animals in
each group. The changes as in the maximum depletion period in-
cluded an increased proportion of slower migrating α-lipoprotein
on acrylamide gel than was found in the continuously chow fed
animal.

A distinct relationship between the proportion of slow migra-
ting α-lipoprotein and serum cholesterol concentration (Fig. 2)
was found, which could be expressed as

% slow α-lipoprotein = 7/8 (choles. concen.-47.5 $\pm$ 8.75).

During the six weeks of protein repletion the total serum protein
concentration of the rats returned to the normal range for rats on
chow diet continuously, being 6.8, 7.3 and 6.7 g/dl for chow-

Table 5.   Change in Body Weight, Serum Cholesterol and Triglyceride of Rats During Protein Depletion and Repletion

	Body Weight, Grams				Serum Cholesterol, mg/dl			Serum Triglyceride, mg/dl		
	Initial	4 wks PFD[1]	10 wks PFD	6 wks CD[2]	Initial Control	10 wks PFD	6 wks CHD	Initial Control	10 wks PFD	6 wks CD
Chow Group N=7	257	344	239	385	74	125	99	80	89	69
% change from prev. level			-30%	+61%		+65%	-27%		range 56-120 +11%	32-95 -19%
% change from start PFD			-30%	+12%		+65%	+23%		+11%	-19%
				6 weeks HED[3]						6 weeks HED
High energy diet group N=10	262	394	244	360	74	115	85	78	73	72
% change from prev. level			-30%	+51%		+55%	-26%		range 37-127 -6%	32-95 0%
% change from start PFD			-30%	-9%		+55%	+15%		-6%	-8%

1 - PFD = protein deficient diet
2 - CD = chow, nutritionally complete diet
3 - HED = high energy, high fat diet
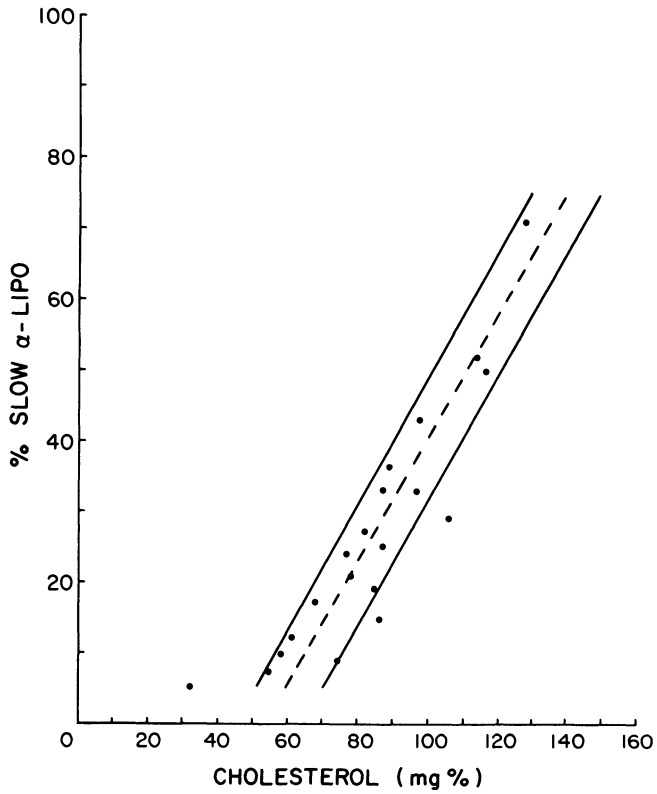
% slow α-lipo = 7/8 (Cholesterol Concentration) - 47.5 ± 8.7

Fig. 2. Relation of slow α-lipoprotein concentration to total cholesterol concentration in rats during protein depletion.

repletion, high energy diet repletion, and continuous chow diet, respectively.

Fatty infiltration of the liver was found in the rats studied after ten weeks of protein deprivation. After six weeks on nutritionally complete diet, rats on the chow diet showed normal hepatic architecture, while those on the high energy, fat diet continued to show fatty infiltration.

RESULTS OF YOUNG RATS PROTEIN DEPLETED WHEN GIVEN ADEQUATE NUTRITION PARENTERALLY

As in the preceding section when rats after protein depletion were returned to a nutritionally adequate program, given in this group of rats (see Methods, Effect of adequate amino acid in Section c parenterally, they showed rapid increase in serum protein and albumin levels. The lipid and lipoprotein patterns of the group receiving the fat free formula became nearly normal, while those on the fat-supplement formula showed significantly elevated serum cholesterol levels. (These studies will be published in detail in a separate publication). Electron microscopic studies on livers of the protein depleted rats showed a marked increase in cytoplasmic fat and smooth endoplasmic reticulum and a marked decrease in rough endoplasmic reticulum. Protein repletion reversed these ultrastructural and biochemical abnormalities (Figs. 3-5).

Discussion

In the protein depleted dogs of this study the serum cholesterol concentration usually increased as the total protein and albumin decreased. The correlation between serum protein and cholesterol concentration, however, failed in some animals in which a low serum protein level was reached where it remained during continuation of the protein-free diet; serum cholesterol reached the highest level when the protein had fallen to the lowest level, then decreased despite continued low protein levels. Variations like these depending partly on duration of protein deprivation may help explain some of the varied effects of protein deprivation on serum lipid levels that have been reported.

The serum lipid and lipoprotein changes associated with deficient protein nutrition vary greatly in the animal or the human being. Thus in Kwashiorkor in which calories are usually adequate but protein deficient β-lipoprotein is reduced and according to some authors cited in the review by Roberts (1980) α-lipoprotein levels are maintained. The changes in lipoprotein pattern are thought to be due to a deficiency of apoproteins for synthesis of VLDL. The lipoprotein changes are associated with fatty infil-
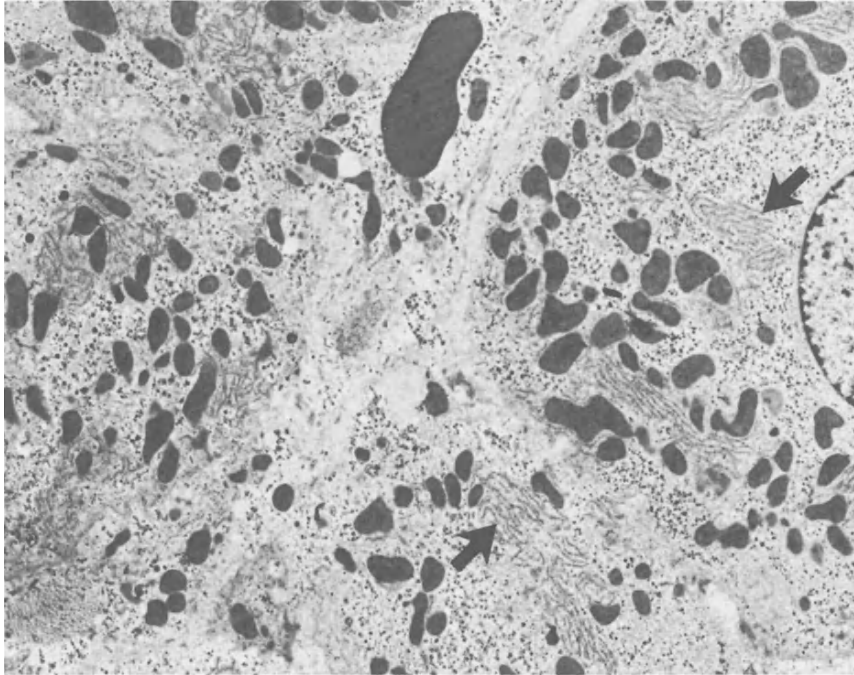
Fig. 3.   Control. Note the abundant, roughly parallel profiles of
          rough endoplasmic reticulum (arrows) in close association
          with the mitochondria (X7020).
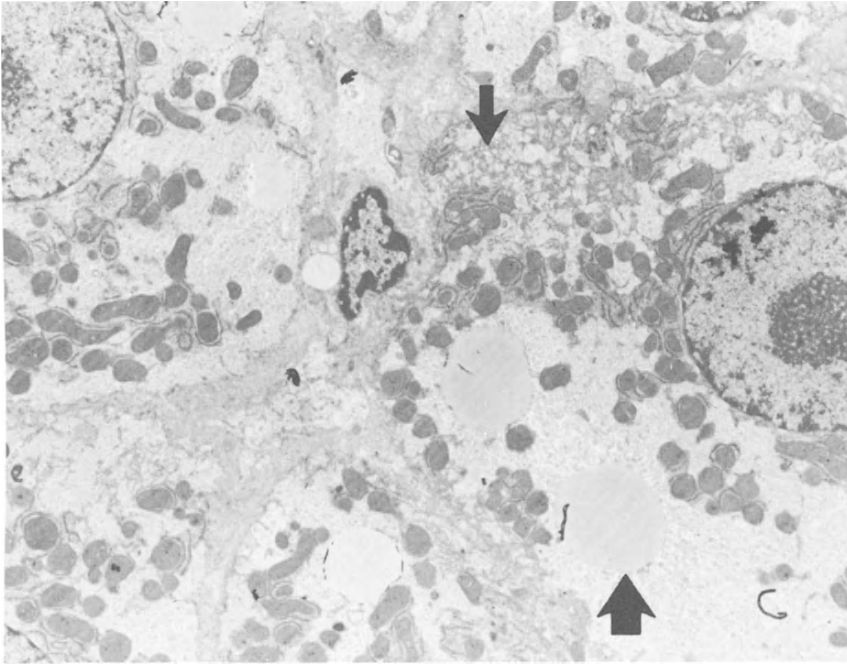
Fig. 4.   Protein depletion. Numerous intracytoplasmic lipid drop-
          lets (large arrow) are present. There is a reduction in
          the amount of mitochondrial associated rough endoplasmic
          reticulum and a proliferation of branching, smooth endo-
          plasmic reticulum (small arrow) compared to the control
          (X7020).
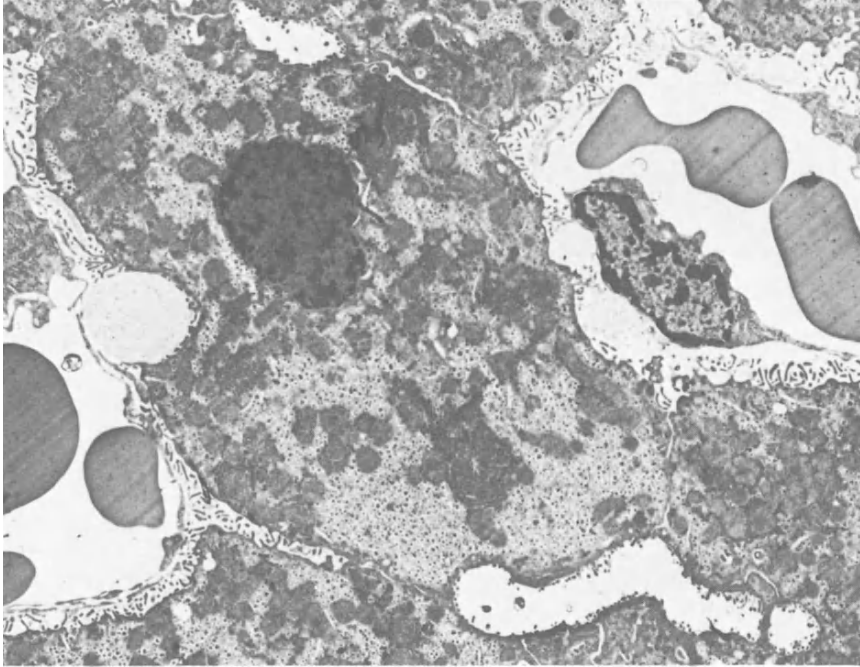
Fig. 5. TPN repletion. Return of ultrastructure to a state
comparable to that of the control (X7020).

tration of the liver and both abnormalities are corrected in the
human being after restoration of adequate protein intake. Lipo-
protein changes are less marked in human subjects with Marasmus
which is the picture of total starvation. Many patients represent
a combination of Kwashiorkor and Marasmus and the changes are
probably determined by which is playing a dominant role. The
relative permanence of HDL levels in our protein depleted dogs,
shown by the fact that HDL, -S 0-5 and -S 5-10, concentrations
were nearly as high during protein depletion as normally, indicates
the preferential use of available protein building materials to
maintain these fractions rather than albumin and some other pro-
teins. It is also possible, however, that the catabolism of HDL
is slower in the protein depleted than in the healthy dog.

The mechanism utilized for the transport of additional
amounts of cholesterol in the protein depleted dog seems to be
similar to that which operates in normal dogs fed large amounts of
added cholesterol (2 g per day). In both, the lipoprotein fractions
with flotation rates between -S 0 and -S 20 (HDL, IDL) are greatly
increased and the -S 20-40, LDL, fraction is also augmented. The -
S 70-400, VLDL and -S >400, chylomicron, are not increased in the
cholesterol fed dog even when the serum cholesterol is as high as
400 mg/dl (Mahley, 1974, 1977). Neither were they increased when
protein supplies for synthesis of plasma proteins (present study)
were limited.

The present studies emphasize the difference in the form of
lipid transport in various species and type of alteration caused
by different pathologic mechanisms. In man (Lewis, 1980) and rats
(Lewis, 1954) with nephrosis, when plasma proteins are low there
is a large increment in VLDL. Also in man and rats low serum
protein levels due to dietary protein restriction have been
reported to cause decrease in TG, VLDL, TC, LDL and HDL (Roberts,
1980).

In nephrectomized dogs maintained by peritoneal dialysis
(Lewis, 1958) serum cholesterol and LDL and IDL fractions usually
increased. Little or no increase in VLDL (-S 70-400) or in -S 40-
70 part of LDL, lipoprotein fractions occurred. The HDL, -S 0-10,
fraction was slightly decreased.

The dog maintains a relatively normal lipoprotein pattern
under various adverse conditions such as protein deprivation and
following nephrectomy when maintained by peritoneal dialysis.
This suggests that in this species the building of lipoproteins is
a "high priority" function. This preferential use of protein
building materials for synthesis of plasma proteins was also
evident in studies of Garrow (1959) using tracer techniques. In
protein depleted dogs he found the turnover rate of proteins to be

less than that of the normals, but found a great increase in the plasma protein specific activity as depletion progressed. This indicated a preferential use of protein for plasma protein synthesis. The preferential use of limited protein supplies for specific purposes has recently been demonstrated in the brain growth of undernourished neonatal rats by Freedman et al. (1980). The undernutrition was produced by feeding the lactating dams a low protein (12% casein) diet. The body weight and brain weights of the pups were 50% and 88% of normal. All areas of the brain were not equally supplied with amino acids as indicated by alterations in serotonin binding and alterations of enzyme activity in norepinephrine and dopamine areas.

Studies by Alusi (1975) on children with protein-caloric malnutrition, showed that of the serum proteins tested all except C_4 and immunoglobulins were lower than in controls. During refeeding the first serum protein to show significant rise was C_3, followed by transferrin, pre-albumin and lastly albumin. Thus in children as in the dogs, protein building materials are selectively used, and albumin is not high on the priority list.

Two conditions in dogs in which transport of cholesterol primarily in α-lipoproteins and β-lipoproteins (-S 20-40) lipoproteins fail are diabetic acidosis and extreme hypothyroidism accompanied by a high-fat, cholesterol rich diet. In these circumstances appreciable amounts of lipids, (i.e., cholesterol, triglycerides, phospholipids) are transported in VLDL lipoprotein (Lewis, unpublished data).

The protein moiety of the canine -S 10-20, (ID) fraction differs from that of the -S 0-10 (α-lipoprotein) as has been demonstrated by the studies of Mahley (1974 and 1979). The apoprotein demonstrated in the -S 10-20 fraction contains apo E, the arginine-rich polypeptide (Shore et al., 1980) which confers on it not only unique physical-chemical properties but also unique biological characteristics. The apo-E containing HDL fraction is capable of binding to the high affinity cell surface receptor of fibroblasts. This receptor also reacts with LDL as was demonstrated by Brown et al. (1976). HDL fractions that do not contain apo E do not have this unique property. Unique chemical properties of the HDL fractions containing apo E include their ability to be precipitated by heparin-manganese, and the relatively high cholesterol content of these fractions.

Clinical and experimental protein-calorie malnutrition are known to be associated with morphological and functional alterations that occur in the liver. A fatty liver in children suffering from Kwashiorkor is a well known feature of the disease and many attempts have been made to define the cause.

Triglyceride is the main lipid that accumulates in the liver of
these patients, while their serum β–lipoprotein concentration is
decreased. Pre-β–lipoprotein may also be low. Alpha-lipoprotein
levels are frequently, but not always, decreased. Reduced hepatic
synthesis of proteins, particularly of some of the apolipoproteins,
may be a major mechanism causing the accumulation of fat in the
liver (Flores et al., 1970). Our electron microscopic studies on
rat livers support this concept. Our results demonstrated that
protein depleted rats showed marked increase in cytoplasmic fat
and smooth endoplasmic reticulum and a marked decrease in rough
endoplasmic reticulum suggesting that protein synthesis is dimin-
ished. Seven days of protein repletion reversed these ultrastruc-
tural and biochemical abnormalities indicating that the process is
readily reversible. In studies on human beings the importance of
nutritional deficiency as a cause of liver injury was stressed by
Tandon et al. (1974). They found that in protein-caloric mal-
nutrition hepatomegaly occurred in six of fourteen patients
studied although liver function studies including bilirubin,
glutamic pyruvic transaminase and alkaline phosphatase were normal.
The mean serum protein concentration was decreased to 4.9 and
albumin to 2.6 g/dl. In all fourteen patients ultrastructural
studies revealed organelle pathology of hepatocytes including
conspicuous changes of rough endoplasmic reticulum and mitochondria.
Repeat studies on seven of the patients after recovery from pro-
tein-caloric malnutrition showed that the ultrastructural abnormali-
ties of the liver were nearly completely reversed to normal.
These observations indicate that the liver changes due to protein
deprivation are reversible. Our EM results agree completely with
this concept. Our studies on the dogs which showed a restoration
of normal serum lipoprotein and protein pattern after restoration
of protein adequate nutrition also suggest an improved hepatic
function. In contrast with protein deficient dogs which showed no
increase in concentration of serum lower density (i.e. -S >40)
lipoproteins some protein depleted rats showed large increments in
these fractions. Nephrotic rats also exhibited increased con-
centrations of serum VLDL and of chylomicron (Lewis and Heymann,
1954).

   In protein malnutrition studies on rats Blanchard et al.
(1979) observed as we have in these experiments that decreases in
serum total protein and albumin are accompanied by an increased
α_1-globulin level. In rats treated by parenteral nutrition after
protein depletion Steiger et al. (1977) noted that fatty livers
persisted if the nutrient solution contained 40% of the non-amino
acid calories as Intralipid [R] *, whereas the hepatic lipids de-
creased promptly when the nutrient solution contained only carbo-

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*Cutter Laboratories, Berkeley, CA

hydrate for supplemental calories.

In total parenteral nutrition of the human being who is nu-
tritionally depleted at the beginning of treatment, one of the
complications which may occur is an essential fatty acid deficiency.
It is manifest by classical clinical signs and the presence of
eicosatrienoic acid in plasma (Richardson and Sgoutas, 1975). In
the protein depleted animal no essential fatty acid deficiency
could occur as the diets given were adequate in essential fatty
acid.

The effect of amino acid diets upon serum lipids in man was
studied by Olson et al. (1970). In the original study (Olson,
1958) in which the diet consisted of 25 g rice and mixed vegetable
protein a fall of 20 percent in the serum cholesterol level was
achieved. Addition of methionine, the limiting amino acid, caused
an increase in serum cholesterol in some but not all of the sub-
jects. In later studies (Olson et al., 1961; Bazzano et al., 1968;
Olson et al., 1964; Olson, 1966) the essential amino acids were
supplied in needed amounts and glutamate was given as the sole
source of nonessential nitrogen. With this combination a very
significant decrease in serum cholesterol was achieved, but was not
observed if glycine replaced glutamic acid in the mixture. In the
study negative nitrogen balance occurred in one dietary period in
which the amino acids and glutamate were omitted. On this diet the
serum cholesterol increased. It was suggested that in this period
necessary nitrogen was obtained by catabolism of body protein.
This was the situation in our animals during protein depletion,
where nitrogen was only available from body protein.

The importance of the quantity and quality of the protein in
the diet in determining serum lipid and lipoprotein levels and the
hepatic lipid levels is not a unique property of protein among
components of foods. The effect of a given level of protein may be
greatly modified by the type of carbohydrate in the diet as was
clearly demonstrated in dietary studies on rats by Cohen and
Teitelbaum (1966). They found a higher incorporation of acetate
into liver fatty acids and cholesterol _in vivo_ and _in vitro_ in rats
fed a sucrose rich diet than in those receiving a high starch diet.
Reduction of the dietary protein from 18 to 11% and replacing the
calories with sucrose in the sucrose diet group caused increased
hepatic fatty acid and cholesterol synthesis. In contrast, in-
crease in starch content of the diet of the starch fed group
caused only an increased hepatic fatty acid synthesis but a de-
creased acetate incorporation into cholesterol.

In a study on young cebus monkeys subjected to diets low in
protein, energy or a combination of protein and energy deficiency,
it was concluded that an energy restriction superimposed on a

limited protein intake did not accelerate evidence of protein
deficiency (Samonds and Hegsted, 1978). High energy diets have
frequently been administered to people following extreme caloric
and protein deprivation. Our finding that restoration of serum
lipid and lipoprotein levels to near normal in the protein depleted
rats was equally effective whether the well balanced chow diet or
the high energy high fat diet was used indicates the validity for
use of such diets in human nutrition. In both the importance of
adequate protein is indicated. A further study is needed, however,
to determine how long a balanced chow diet would be needed to re-
duce hepatic lipid levels of the high energy diet animals to normal.

Studies of two men who were on a raft for fifty-six days with-
out food, and ingesting only suboptimal amounts of water during the
last twenty-six days of the trip showed some abnormality of liver
function for a short period during refeeding (Lee et al., 1977).
In our study rats receiving a high energy diet for refeeding showed
fatty infiltration of the liver while those receiving a conventional
rat chow diet low in fat had histologically normal hepatic archi-
tecture. These results raise the possibility that careful consid-
eration of the type of diet during refeeding after protein depriva-
tion may be of importance. The fact that normal liver function
ultimately was observed in the Lee study (1977), however, is indi-
cative of the great recuperative power of the liver.

The changes in serum lipids and lipoproteins caused by protein
deprivation observed in the dogs and rats of this study were ob-
viously affected by many factors including age of animal, duration
of deprivation of protein and composition of the diets ingested.
These results emphasize the complex nature of studies involving
dietary protein deficiencies and why experts still after years of
investigation find large gaps in knowledge about malnutrition and
why "the role of neither protein nor calories is clearly defined"
(Hegsted, 1978).

SUMMARY

The ultracentrifugal and starch-gel electrophoretic patterns
of the serum lipoproteins of the normal dog have been described
and contrasted with those of the protein-depleted animal. The
serum cholesterol and lipoprotein concentrations of dogs were
usually increased during protein depletion produced by plasma-
phoresis and feeding of a protein-free diet. The increased choles-
terol was transported primarily in -S 10-20 and -S 20-40 lipopro-
tein fractions, with some increase in the more dense -S 0-5 and
-S 5-10 lipoprotein fractions. There was no increase in concentra-
tion of VLDL (-S > 40).

The ability of the protein depleted dog to maintain high concentrations of protein-rich lipoproteins was noteworthy.

In protein depleted, old rats, there was some increase in the concentration of very low density -S 70-400 and -S 40-70 lipoprotein fractions.

Young mature rats on a protein deficient diet showed significant alteration in their serum lipoprotein patterns. The concentration of α-lipoprotein of fast electrophoretic mobility decreased, while that of slower mobility increased. The concentration of β-lipoprotein also increased. Livers of both young and old, protein depleted rats showed fatty infiltration. Serum lipoprotein and hepatic structure returned toward normal after the young rats were returned to a nutritionally complete diet.

It is concluded that the serum lipoprotein patterns of both dogs and rats are altered during prolonged periods of protein deprivation. The changes are reversible, the patterns returning toward normal with a nutritionally complete ration. Changes in hepatic structure were noted in the protein depleted rat; these changes also were reversible.

ACKNOWLEDGEMENTS

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DIETARY FIBER AND DISEASE

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INTRODUCTION

Dietary fiber is defined as plant cell wall materials which
are not subject to hydrolysis by human digestive enzymes (Trowell,
1972b). This group of materials includes a large number of poly-
saccharides, including cellulose, hemicellulose, pectins, gums,
as well as lignin. In plants these polysaccharides serve as either
a storage form of carbohydrate or as structural components of the
plant. The physical characteristics of these polysaccharides and
lignin in relation to dietary fiber have been expertly reviewed by
others (Cummings, 1976; Southgate, 1976).

In spite of their almost total unavailability in humans, the
components of dietary fiber play an important role in human nutri-
tion, both normal and as possible preventative factors in several
disease states of major proportion in the Western word (Burkitt,
et al., 1974). Interest in the role of dietary fiber in human
nutrition has been rekindled in recent years as a result of these
epidemiologic and experimental observations relating this component
of the diet to the incidence of a large number of diseases. This
review will examine the evidence for the relationship of dietary
fiber to three specific disease states (atherosclerosis, colon
cancer and diabetes) in an attempt to clarify the current status
of our understanding concerning the role of dietary fiber in
causation of these diseases.

ATHEROSCLEROSIS

The relationship between intake of a high, complex carbohydrate
diet and lower incidence of coronary heart disease in humans was

observed by Walker et al. (1961). They found that, in addition to the Bantu, whites who consumed a diet similar to that of the Bantu had an extremely low incidence of coronary heart disease as evidenced by electrocardiographic examination. This diet was described as very high in carbohydrate (1-1.5 pounds of dry cereal per day) and low in animal fat and protein. They also noted that white prisoners who consumed large amounts of cereal products had an extremely low incidence of death from coronary disease.

Trowell (1972c) brought attention to these studies and noted other reports (Antonis and Bersohn, 1962) which dealt with bile acid excretion when Western diets were compared with high-fiber African diets. High-fiber diets resulted in increased bile acid excretion which was suggested by Trowell as a possible mechanism for decreased cholesterol levels by a mechanism similar to that observed with bile acid sequestrants. Trowell (1972a) also reported concomitant changes in the incidence of heart disease and diverticular disease in Great Britain during the previous 40 years. Periods during the second World War when dietary fiber intake was increased were accompanied by a slowing of the increase in incidence of both diseases. In addition other populations which eat diets high in dietary fiber have lower serum cholesterol levels and a decreased incidence of coronary heart disease (Trowell, 1972a).

Most of this epidemiological work was brought together in a report by Burkitt et al. (1974). The incidence of a large number of diseases prevalant in Western civilizations was compared with the incidence of these diseases in lesser developed populations. Dietary fiber intake was discussed as a major determinant in these differences. Changes in nutrient availability, transit time, intracolonic pressure, intestinal flora, and bile salt metabolism were suggested as mechanisms responsible for the changes in incidence of disease. Ischemic heart disease was described as "virtually unknown" in native African populations while being responsible for one-third of all deaths in the United States.

Studies in experimental animals had also indicated an involvement of dietary fiber in cholesterol metabolism and experimental atherosclerosis. Portman (1960) reported increased cholic acid excretion and reduced half-life of cholic acid in rats fed a commercial ration compared to those fed a semi-purified diet with cellulose added for fiber. His experiments also found that some fraction of the grain component of the commercial ration was responsible for these changes.

In rabbits, Kritchevsky and Tepper (1968) found that a component of commercial rations other than lipid resulted in a resistance to high saturated fat levels which, in a semi-purified diet, resulted in cholesteremia and atherosclerosis. Cookson et al. (1967) had also reported a regression of dietary cholesterol induced

cholesteremia in rabbits using a diet supplemented with large
amounts of alfalfa. Atheroma was also shown to be prevented by
some sources of dietary fiber when added to an atherogenic semi-
purified, cholesterol-free diet in rabbits. Wheat straw and peat
appeared to reduce the cholesteremic and atherogenic effects of the
diet with cellophane spangles added for bulk (Moore, 1967).

Bile acid metabolism and cholesterol absorption had also been
shown to be altered by some sources of dietary fiber. Alfalfa
decreased absorption of an oral dose of radioactive cholesterol
and increased excretion of bile acids synthesized from this
radioactive precursor (Kritchevsky et al., 1974). Pectin had also
been reported to increase fecal bile acid excretion (Lin et al.,
1957; Leveille and Sauberlich, 1966). Eastwood and Boyd (1967)
began the investigation of a mechanism for these changes in ex-
cretion when examining the distribution of bile salts along the
intestine. They observed a significant quantity of bile salt
associated with the solid material in the small intestine and not
in free solution. Later experiments (Eastwood and Hamilton, 1968)
indicated an ability of dietary fiber sources to adsorb bile salts
in vitro. Experiments indicated that lignin was an important
component of dietary fiber in bile salt adsorption and that the
adsorption was hydrophobic in nature. Others (Kritchevsky and
Story, 1974; Story and Kritchevsky, 1976) have also observed
adsorption of bile salts by a large number of dietary fiber sources
and components.

Subsequent experiments in man and animals have investigated
the cholesterol lowering capacity of dietary fiber and many of its
components and have examined several possible mechanisms for the
observed changes. In experimental animals, lignin has been shown
to reduce cholesterol levels or prevent their increase in rats due,
in part at least, to increases in bile acid excretion (Judd et al.,
1976; Story et al., 1977). This correlated well with the in vitro
finding of bile acid adsorption in vitro. In man lignin has not
been extensively examined but has been shown to reduce human serum
cholesterol levels by some workers (Thiffault et al., 1970) and
has resulted in significant increases in the hands of others
(Londner and Moller, 1973).

Cellulose has been used extensively in both animal and human
studies and, as evidenced by its lack of interaction with bile
salts, has not been found to result in many changes in cholesterol
metabolism. In rats cellulose results in little change in choles-
terol levels of liver and/or serum (Looney and Lei, 1978). When
fed with cholesterol, cellulose does not prevent the liver accumu-
lation of cholesterol in rats (Tsai et al., 1976) but reduces the
accumulation in comparison to a fiber-free diet (Story et al.,
1977). In rabbits, cellulose is an important part of a cholesterol-
free, semipurified atherogenic diet as shown by the lack of

effectiveness of the diet when other sources of dietary fiber
are used (Hamilton and Carroll, 1976; Kritchevsky et al., 1977).
In man a similar lack of effect has been observed at reasonable
levels of administration (Eastwood et al., 1973).

Hemicellulose has been reported as an effective adsorbant of
bile salts (Birkner and Kerm, 1974) but studies in man and animals
have been hindered by a lack of availability of pure hemicellulose.
Psylium seed has been reported to prevent cholesterol accumulation
in cholesterol fed rats (Beher and Casazza, 1971) and to lower
serum cholesterol levels in man (Garvin et al., 1965; Forman et
al., 1968). Fecal bile acid excretion was reported to increase in
both cases.

Recent attention has focused on several soluble polysaccharides
and their cholesterol lowering effects. Pectin has long been re-
ported to lower serum cholesterol in humans (Keys et al., 1961).
In experimental animals many workers have reported reductions or
lack of accumulation of cholesterol in rats (Ershoff, 1963; Tsai
et al., 1976; Chang and Johnson, 1976; Judd et al., 1976; Story
et al., 1977), rabbits (Berensen et al., 1975), and chickens
(Fisher et al., 1965; Fahrenback et al., 1966). Guar gum also
reduces cholesterol levels in experimental animals (Tsai et al.,
1976).

A recent review (Truswell, 1979) examined 12 experiments using
a variety of levels of pectin supplementation (2-35 g/day) in man.
Significant reductions in plasma cholesterol were reported in all
but two experiments and in those lower, but not significant, levels
were also reported. Examination of the mechanism involved (Kay
and Truswell, 1977) indicated an increase in both neutral and acidic
steriods in the feces of the pectin-fed subjects. Guar gum also
reduces plasma cholesterol levels in man (Jenkins et al., 1975).
A recent experiment in which guar was incorporated into crispbread
and fed to hyperlipidemic subjects (Types II and IV) at a level of
13 g of guar/day for 8 weeks (Jenkins et al., 1980). The guar
treatment reduced plasma cholesterol levels 13%, with high density
lipoprotein cholesterol (HDL) levels remaining constant.

Wheat bran, another source of dietary fiber which is used
extensively by humans, has been extensively examined for cholesterol-
lowering capability. A summary of these reports (Truswell and Kay,
1976) indicated a lack of effect of widely varied dose (14-100 g/day)
on plasma cholesterol or triglyceride levels in man. More recently
hard red spring wheat bran (39 g/day) has been shown to lower plasma
cholesterol in humans (Munoz et al., 1979). The earlier studies
used the more common white wheat bran. There are no apparent dif-
ferences in composition between the two sources of bran, but some
difference is apparently responsible for the observed difference
in function.

Another source of dietary fiber for humans which has recently
been examined more thoroughly is oats, and more specifically, oat
bran. The cholesterol-lowering effects of whole oats (rolled) has
been observed in experimental animals (DeGroot et al., 1963; Fisher
and Griminger, 1967) and man (DeGroot et al., 1963; Lukyen et al.,
1965). Recent evidence (Chen and Anderson, 1979) indicates that
oat bran reduces low density lipoprotein (LDL) cholesterol while
increasing HDL cholesterol in the rat. In man they have also
reported a selective lowering of LDL (Anderson and Chen, 1979).

The effectiveness of dietary fiber in lowering risk of athero-
sclerotic heart disease has been examined primarily in relation to
its ability to lower blood cholesterol levels. The first mechanism
to attempt to explain the ability of some sources of dietary fiber
to lower cholesterol levels dealt with the ability of these dietary
fibers to increase fecal steroid excretion. Adsorption of bile
salts by dietary fiber increases their excretion by inhibiting
reabsorption. In addition adsorption of bile salts in the small
intestine also interferes with cholesterol absorption which results
in increased fecal neutral steroid excretion from both dietary and
biliary sources. The net result of these changes is to increase
fecal steroid excretion sufficiently so as to decrease body pools
of cholesterol. Reduction of serum cholesterol is considered a
decrease in risk of developing coronary heart disease.

Recently a second possible mechanism of action for reduction
of risk has emerged. This involves the observation that reductions
in serum cholesterol resulting from changes in dietary fiber appear
selectively in the LDL fraction with no changes or possibly increases
in HDL cholesterol levels. Since LDL cholesterol is positively
correlated with atherosclerosis and HDL cholesterol is negatively
correlated, these selective changes seem to offer a second positive
effect of dietary fiber on cholesterol levels. Mechanisms for the
specific changes in LDL and HDL have not been elucidated at this
time.

COLON CARCINOGENESIS

An epidemiological association between dietary fiber intake and
cancer of the colon was enumerated and a hypothesis concerning the
mechanism for this relationship offered by Burkitt (1971). The
relationship began as a comparison of the high fiber, low cancer
incidence populations of Africa with the low fiber high incidence
populations of the Western world. The mechanism derived from
several observations concerning the effects of high dietary fiber
diets. The amount of dietary fiber consumed is related inversely
with the transit time of material through the intestine (Walker,
1961). In addition, differences in fecal bacteria in relation to
the level of dietary fiber in the diet had been observed (Hill et
al., 1971; Aries et al., 1971). Diets low in dietary fiber

resulted in bacteria with a greater ability to 7α-dehydroxylate
bile acids and likewise a larger amount of secondary bile acids
(deoxycholic and lithocholic), the products of this 7α-dehydroxyla-
tion. The observation that deoxycholic acid could be converted to
a potent carcinogen in the laboratory (Haddow, 1958) resulted in
speculation concerning the possibility of these reactions taking
place in the colon. Using these facts, Burkitt hypothesized that
low fiber diets resulted in increased concentrations of the poten-
tially carcinogenic secondary bile acids in the colon and, as a
result of increased time of exposure as a result of slowed transit,
these bile acids would have more of a chance to interact with the
intestinal epithelium.

The relationship of transit time with the incidence of colon
cancer in humans has not been resolved. Burkitt et al. (1974)
found a strong inverse correlation between transit time and fecal
weight and suggested a relationship between increased stool weight
and decreased transit time and a lower incidence of colon cancer.
Other studies have indicated that transit time is not as well re-
lated. Eastwood et al. (1976) noted that, in spite of a higher
incidence of constipation in females, the occurrence of colon cancer
is slightly lower in females than in males. Glober et al. (1974)
observed an almost two-fold greater transit time in Caucasian
Hawaiians than in those of Japanese descent while colon cancer rates
were very similar. The role of transit time in susceptibility to
colon cancer remains to be fully understood.

Bile acids have become a major focus of study in understanding
differences in susceptibility to colon cancer caused by changes in
diet. Subsequent to the studies implicating bile acids mentioned
above, several workers have more thoroughly examined the role of
bile acids in colon carcinogenesis. Hill and Aries (1971) first
suggest this relationship by correlating fecal bile acid concentra-
tions with the incidence of colon cancer in several countries.
Colon cancer patients were also found to have a higher concentration
of bile acids in their feces than non-cancer patients (Hill et al.,
1975).

Animal studies have also indicated a role of bile acids as
co-carcinogens. Narisawa et al. (1974) used intrarectally instilled
N-methyl-N'-nitro-N-nitrosoguanidine as a carcinogen and found that
lithocholate and taurodeoxycholate applied repeatedly after the
carcinogen resulted in more tumors than when they were absent.
These two bile acids did not cause tumors when administered alone.
Lithocholic has also been found to be co-mutagenic with two car-
cinogens in the Ames assay (Kelsey and Pienta, 1979). Lithocholic
was also mutagenic in the hamster embryo cell transformation assay
(Silverman and Andrews, 1977). Increasing concentration of colonic
bile acids also increases susceptibility to chemical carcinogens
in rats when these changes are caused by drugs such as choles-

tyramine (Nigro et al., 1973), by transplantation of the bile duct (Chomchai et al., 1974), or by feeding a high fat diet (Nigro et al., 1975).

Experiments examining directly the effects of various sources of dietary fiber on the susceptibility of rats to chemical carcinogens have had mixed results. Wheat bran generally has been shown to decrease the incidence of colon tumors in this model. Wilson et al. (1977) fed 20% wheat bran and either beef fat or corn oil and administered dimethylhydrazine (DMH) subcutaneously. Fewer animals had tumors with either dietary fat when fed wheat bran, although the number of tumors per tumor bearing animal was slightly higher. Fleiszer et al. (1978) added 28 g wheat bran per 100 g diet for rats and also administered DMH and also found a much smaller number of animals with tumors in the bran fed group. Nigro et al. (1979) and Watanabe et al. (1979) also observed reduced tumor incidence when wheat bran was fed to animals given azoxymethane (AOM) injections to induce tumors. Nigro et al. (1979) also observed that cellulose and alfalfa resulted in fewer tumors than a fiber-free diet when a low-fat diet (10%) was fed but observed little difference with a high-fat (35%) diet. Using an intrarectally administered carcinogen (methylnitrosourea) Watanabe et al. (1979) found that alfalfa (15%) increased tumor incidence while wheat bran (15%) reduced incidence slightly and pectin (15%) dramatically.

These data seem to be consistent with the above discussion of bile acid concentration and carcinogen susceptibility. Those sources of dietary fiber which adsorb bile salts, e.g. alfalfa, result in smaller reductions or increases in tumor incidence while those which do not bind bile acids but dilute the feces by holding water result in lower tumor incidence. Nigro et al. (1979) measured bile acid concentration in the feces of animals used in the above experiments and found a direct relationship between bile acid concentration and susceptibility to colonic tumors induced by AOM. Thus experimental evidence to date would indicate an involvement of dietary fiber in colon carcinogenesis mediated, at least in part, through its effects on bile acid excretion.

DIABETES MELLITUS

Trowell (1973, 1974) reported an epidemiologic association between consumption of complex carbohydrate diets, especially high in dietary fiber, and the incidence of diabetes mellitus. He correlated incidence of diabetes mortality in women in England and Wales with the crude fiber content of flour during the years prior to and during the second World War. While the crude fiber content of flour increased during the war and sugar intake decreased the number of deaths decreases. Cleave (1966) had also associated the concomitant increase in simple carbohydrates and decrease in

complex carbohydrate intake with increase in a number of diseases including diabetes.

Several experiments in man have indicated the validity of these arguments. Kiehm et al. (1976) utilized a high carbohydrate, high plant fiber diet in patients with diabetes. Reductions in fasting glucose and in insulin or oral hypoglycemic doses was observed in all patients. Anderson et al. (1979) have recently extended these experiments to a larger number of patients all of whom were receiving exogenous insulin. The diet contains 70% of kilocalories from carbohydrate, 71% of which were considered "complex" carbohydrates, 9% of kilocalories from lipid and 21% of kilocalories from protein. Weight did not change significantly while dependance on insulin was reduced in all cases and was eliminated in all cases where 20 units/day or less was required prior to diet change. Plasma glucose concentrations, both fasting and post-prandial, were reduced and urinary glucose was also reduced. Serum cholesterol was also reduced significantly.

Jenkins et al. (1977) have reported lower post-prandial glucose and serum insulin levels in normal subjects to which guar gum and pectin were added to the test meal. Subsequent studies (Jenkins et al., 1978) have indicated that viscosity of the added dietary fiber is a critical characteristic with the more viscous materials being more effective in altering glucose metabolism.

Similar findings have been reported using test meals (60 g carbohydrate) given to normal subjects as apples, apple puree or apple juice (Haber et al., 1977). The more complex the associated carbohydrate the lower the peak plasma glucose level and the smaller the hypoglycemic rebound after this peak level. Plasma mortality levels were also reduced when apples or puree were consumed, compared to apple juice. These changes in insulin response appear to be related to an alteration in site of absorption of glucose to a more distal location which would alter secretion of gastric inhibitory polypeptide (Thomas et al., 1977) and, correspondingly, reduce insulin secretion. The long-term effects of these changes in insulin secretion appear to reduce the insulin requirement as is evidence by the work of Anderson discussed above.

CONCLUSIONS

The study of the involvement of dietary fiber in both normal and disease oriented nutrition has evolved as an important part of nutrition. Use of various sources or components of dietary fiber for prevention or alteration of risk of atherosclerotic heart disease or colon cancer and in treatment of diabetes have been examined. In all cases a great deal of variation is found in the effects observed with various dietary fiber sources. However theoretical mechanisms of the involvement of dietary

fiber have been proposed and experimental data seem to indicate
that many of these mechanisms are valid with selected dietary fibers.

Atherosclerosis in experimental animals and selected risk
factors for atherosclerosis in humans are modified by some com-
ponents of dietary fiber, i.e. pectins, lignin and some gums.
These components appear to alter cholesterol metabolism by increas-
ing excretion of bile salts and/or neutral steroids in the feces,
a mechanism suggested very early in the study of dietary fiber
(Eastwood, 1969). Data presented appear to support the above
mechanism but do not appear to explain more recent observations
concerning changes in lipoprotein cholesterol levels.

The mechanism of involvement of dietary fiber in colon cancer
is also tied to its interaction with bile acids. Increased con-
centration of bile acids in the colon apparently results in increased
susceptibility to chemical carcinogens. The effects of various
dietary fibers seems to be related to their ability to increase
or decrease bile acid concentrations by adsorption of bile acids
or absorption of water, respectively. The role of altered transit
time or direct effects on carcinogen absorption or activation have
not been fully examined.

Diabetus mellitus can be treated using a high complex car-
bohydrate and low fat diet. This diet has the added benefit of
lowering serum cholesterol levels in a group of people already at
a higher risk for heart disease. Alteration of the absorption
site of glucose appears to alter the hormonal response to glucose
and improve the metabolism of the absorbed nutrients.

In all cases, changes in absorption of nutrients or other
materials is an integral part of the effects of dietary fiber on
the parameters in question. Dietary fiber components, either
through direct adsorption, dilution, changes in transit speed,
or alteration of the absorptive surface change the amount of
material absorbed or the location in the intestine of absorption.
One or both of these changes can obviously have marked effects on
the eventual metabolic fate or effect of the absorbed constituent.

It would seem essential to gain a better understanding of the
effects of these changes in absorption. Knowledge of physical
properties of components of dietary fiber and resultant physiolo-
gical changes will allow selection of dietary fiber for specific
desired changes without other unwanted side effects.

Dietary fiber has progressed from one of the least examined
parts of our diet to one of faddish popularity. Our ability to
ascertain answers to many of the above questions and thus gain an
understanding of the role of dietary fiber in nutrition will
eventually place dietary fiber in proper perspective in relation

to other diet components. Dietary fiber is an important part of
our overall nutrition, but until we do understand its place in
nutrition, its use as a therapeutic agent must be limited to a
few well examined situations.

ACKNOWLEDGEMENT

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SOME MANIFESTATIONS OF MALABSORPTION IN DISEASE

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INTRODUCTION

The term malabsorption has come to include both defective di-
gestion and absorption. All ingested nutrients, i.e., fat, carbo-
hydrate, proteins, minerals, H_2O, and vitamins, normally enter the
body via the gastrointestinal tract. It must be remembered that
the absorbing surface of the digestive tract is not a one-way
street. Therefore, in addition to oral food intake, a significant
load of 'endogenous nutrients' is added to the intestinal contents
of the alimentary canal. Complex nutrient molecules of both exo-
genous and endogenous origin undergo stepwise degradation to absorb-
able form and are transported into the intestinal cell also in a
stepwise manner. Consideration of these steps and appreciation
that absorbed nutrients exit from the intestinal cell, via either
the portal vein or the lymphatic system, are essential to under-
standing malabsorption.

Equally important, though not as well appreciated, are the
nutrient interactions and discriminations which occur not only in
the G.I. tract but in other tissues. It would be highly desirable
from the laboratory point of view to be able to relate specific
tests to specific problems. However, malabsorption problems fre-
quently present as multiple nutrient deficiencies of chronic syn-
dromes, seldom as deficiencies of single nutrients, which have been
characterized so well by the nutritional biochemists of the 40's
and 50's.

REVIEW OF ASSIMILATION OF NUTRIENTS

Ingested food has been subjected to maceration, acidification, denaturation, and dilution by the time it leaves the stomach. Little digestion occurs until it is exposed in the small intestine to pancreatic enzymes and, in the case of dietary fat, solubilized with conjugated bile acids. The final steps in digestion of carbohydrates and protein occur on and within intestinal epithelial cells yielding simple compounds to be removed via the portal system. The micelles formed within the intestinal lumen from bile salts, fatty acids and β-monoglycerides disaggregate at the epithelial surface. The bile salts remain within the lumen, available for reuse, while lipid components readily pass the lipophilic brush border membrane. Products of fat digestion, long chain fatty acids ($>C_{10}$) and their mono and diglycerides, must be modified to triglycerides, packed with other lipids within a lipoprotein envelope to form chylomicra before they can exit from the cell to be carried away via the lymph. Short chain fatty acids (called medium chain in medical literature) are absorbed directly without modification and removed via the portal vein.

Interference with any of these steps essential to digestion, absorption and transport usually results in malabsorption with accompanying steatorrhea and diarrhea. Dehydration due to electrolyte and water loss may become rapidly critical, particularly in infants and young children because of their high extracellular water. In addition there is evidence that the bowel of the infant is more permeable and susceptible to osmotic loads than the bowel of adults (Younoszai et al., 1978).

ABNORMALITIES ASSOCIATED WITH MALABSORPTION

Most abnormalities associated with malabsorption are readily classified into those of intraluminal phase, mucosal cell transport and intestinal lymphatic transport of fat. Each of these plus an unclassified category will be considered separately.

Intraluminal Phase

Hydrolysis of dietary triglyceride by pancreatic lipase must precede normal absorption. Lipase at pH 6 to 7 in the presence of conjugated bile salts solubilizes the lipid in the form of monoglyceride, fatty acids and fat soluble vitamins into micelles. A defect in any one of these steps leads to defective fat absorption which is evident by an increased excretion of fecal fat (steatorrhea).

Along with malabsorption of fat, one expects malabsorption of fat soluble vitamins which may be seen in increased prothrombin time where vitamin K is not absorbed, and in hypocalcemia and tetany

where vitamin D deficiency occurs resulting in decreased calcium absorption. Formation of insoluble calcium soaps of unabsorbed fatty acids may also interfere with calcium absorption.

Bacteria in the colon may act upon the increased amount of fat entering the colon to produce hydroxy fatty acids which are irritants and cathartic and further aggravate the diarrhea and decrease transit time (Soong et al., 1972).

Estimation of serum carotene is a simple, commonly used laboratory test for fat malabsorption.

Apparent pancreatic insufficiency may occur from a number of causes such as a low pH in the duodenum which irreversibly inactivates lipase. An example appears in the Zollinger-Ellison syndrome where there is grossly excessive secretion of gastric HCl (Shimoda et al., 1968). Surgical alterations may alter the relationships between gastric emptying and pancreatic and biliary secretion and cause malabsorption. Subtotal gastric resection and gastrojejunostomy divert food stuffs from the normal pathway in the upper small intestine and thus blunt or even eliminate the humoral stimulus for pancreatic secretion which occurs in response to the presence of chyme in the upper small intestine. Further, mixing of bile salts, pancreatic juice and chyme may be inadequate, and, with a probable decrease in transit time, contribute to malabsorption.

Diagnostic criteria for cystic fibrosis are relevant to this classification. In 1970, we (Jones et al., 1970) determined the frequency distribution of sweat sodium values in a large group of patients of varying ages including some with vague G.I. problems. The analysis yielded three groups: those with clearly elevated sweat sodium, diagnosed as having cystic fibrosis; those with normal sweat sodium, diagnosed as free of cystic fibrosis and those with sweat sodiums in between in a gray area. Some of the patients in the gray area presented with G.I. symptoms suggesting that the intermediate sweat sodium concentration could have been a positive for cystic fibrosis. di Sant'Agnese and Davis (1979) reviewed symptoms in 307 patients with cystic fibrosis 18-47 years of age. They found pulmonary disease in 97% of the patients and 95% were identified as suffering from pancreatic insufficiency, but were "seldom symptomatic although steatorrhea and azotorrea were massive". Although many physicians were reluctant to classify such adult patients as having cystic fibrosis, it is now apparent that many patients survive cystic fibrosis into adulthood and have malabsorption as a result of pancreatic fibrosis. The message, here, is that sweat sodium or chloride is an appropriate test for adults and may be essential in making the correct diagnosis of malabsorption.

Synthesis of bile acids from cholesterol and their conjugation occurs in the liver. The bile salts are reabsorbed in the distal

ileum, and patients with diseased or surgically removed small
bowel will suffer from a reduced bile acid pool and subcritical
concentration of conjugated bile acids (1-2 mM) in the upper je-
junum. Thus fat malabsorption is the normal consequence of a
diseased liver and of a diseased or absent ileum with reduced bile
acid pool.

Malabsorption caused by the contaminated small bowel syndrome,
CSBS, is classified as a intraluminal problem. In that it presents
complications in a number of the situations in other classifica-
tions, it will be addressed later.

Mucosal Cell

Diseases in which specific abnormalities in mucosal cell
transport occur include cystinuria, Hartnups, disaccharidase de-
ficiencies, and monosaccharide transport defects. Those genetic
diseases which relate to amino acid assimilation support the cur-
rently accepted concept that peptides are hydrolyzed after uptake
from the intestinal lumen. Glycine is absorbed more readily, as
evidenced by the more rapid rise and greater concentration of
plasma glycine, when consumed as a di- or tripeptide than as the
free amino acid (Craft et al., 1968). Oral loads of free tryptophan
in the Hartnups' patients are poorly absorbed (Asatoor et al.,
1970). However, in both Hartnups and normals, tryptophan in pep-
tides and proteins is readily absorbed and confirms that the absorp-
tion process involves two steps.

Similar experiments on the absorption of cystine and dibasic
amino acids by the cystinuric yielded similar data (Milne et al.,
1961). In fact, ingestion of L-cysteine caused plasma levels of
cystine to increase more in cystinurics than in normals. However,
cystine absorption per se appears to be more complex and to involve
reduction to cysteine during transport into the cell. These genetic
disease of amino acid transport create no gastrointestinal problem
for the patient. As the transport defect is also in the kidney,
the patient loses the amino acids in the urine. Cystinurics get
kidney stones; some Hartnup's patients appear to lose enough tryp-
tophan to have symptoms of niacin deficiency (Jepson, 1972).

Patients with disaccharidase deficiencies do have intestinal
symptoms. The dietary carbohydrate is not hydrolyzed, not absorbed
and remains in the G.I. tract. Water is retained by the osmotic
effect of the unabsorbed disaccharide. The bacteria of the lower
tract metabolize the disaccharide and produce organic acids and
gas, resulting in bloating, distention discomfort and diarrhea.
Lactase deficiency is the most common and most thoroughly studied,
and it does occur in children. Barr and coworkers (1979) brought
attention to the role played by lactose intolerance in chidren with
recurrent abdominal pain.

The lactose-breath H_2 test has been used in a number of these studies. Newcomer et al. (1975) concluded from a prospective study of 25 lactose deficient subjects that breath H_2 is the method of choice for screening large populations for lactase deficiency. It is sensitive, non-invasive and simple to perform but requires a gas-liquid chromatograph for the determination. The lactose breath H_2 test was reported by these investigators to compare very favorably with three other indirect tests, $^{14}CO_2$ from $I-^{14}C$ lactose and increase in plasma glucose after an oral lactose load and the rise in plasma galactose after lactose and ethanol were given together.

Two conditions occur in children, primary alactasia and severe lactose intolerance (Lifshitz, 1966 and Berg, 1969). Lactose is found in the urine in both cases and can be identified by high voltage electrophoresis or thin-layer chromatography. Glucose-galactose malabsorption which presents during the first week of life is an example of a specific defect in transport of glucose unrelated to Na transport. Na transport is normal in this condition. The disease presents as diarrhea and has been diagnosed by identification of the two monosaccharides in feces (Menzies and Seakins, 1974).

Vitamin B_{12} deficiency has no G.I. symptoms per se; it may be due to a lack of intrinsic factor which is usually accompanied by achlorhydria. The vitamin is normally actively absorbed in the ilieum, and resection or disease can eliminate active absorption.

Non-specific abnormalities of mucosal cell transport include gluten sensitive enteropathy, tropical sprue, and effects of radiation and drugs. The gut is very sensitive to radiation and drugs because of the high rate of turnover of the mucosal epithelium. A characteristic flattening of the villi is seen in nontropical sprue. The mucosal structure obtained by peroral biopsy shows flattening of villi in other diseases too (Katz and Grand, 1979) with similar effects to the patient. The lack of normal structure results in loss of mucosal hormones, enzymes, and specific absorptive capacity and, thus, may interfere with absorption of fat, carbohydrate, and protein and, in fact, all nutrients.

Very complex nutritional problems are seen in Crohn's disease of the small bowel which almost always involves the ileum in segmented areas but also involves the jejunum and may be diffuse in its most severe form. Not only does granulomatous infiltration of the ileum and jejunum depress the ability of the columnar cell to absorb multiple nutrients, but intestinal lymphatics are extensively involved, severely impairing fat transport and causing fat to remain in the lumen (Donaldson, 1978). Fistula formation is common. Where enterocolic fistulas occur, there is recirculation of bowel contents with stasis and bacterial overgrowth into the small bowel, leading to deconjugation of bile salts and less effective

solubilization of lipids for absorption. Whatever abnormalities
lead to fat malabsorption, the resulting diarrhea and steatorrhea
may cause demineralization of bone and hypocalcemic tetany due to
vitamin D malabsorption and deficiency followed by decreased
calcium absorption and to hypoprothrombinemia due to vitamin K
malabsorption. Abnormal xylose tolerance tests also demonstrate
the reduced capacity for absorption of water soluble nutrients such
as iron, calcium, vitamins and others. Clinically, megaloblastic
anemia from depressed folate absorption occurs as does iron de-
ficiency anemia from depressed iron absorption. Persistent loss
of small amounts of blood via the intestinal wall may contribute to
iron deficiency anemia. Surprisingly, with a diseased ileum, the
vitamin B_{12} absorption is usually good unless bacterial overgrowth
interferes. Weight loss due to failure to assimilate adequate
calories is characteristic. Protein losing enteropathy may be
severe leading to hypoalbuminemia. The diarrhea and steatorrhea
and presence of bile salts in the colon contribute to the develop-
ment of hyponatremia, hypokalemia and dehydration. Also contribut-
ing to final inanition from lack of adequate absorption are anorexia
and fear of eating because of expected aggravation of crampy pain
and distention. Absorptive capacity may be further impaired by
surgical treatment to remove varying amounts of diseased ileum and
jejunum.

A relatively rare disease often affecting the terminal ileum
and producing diarrhea is the carcinoid syndrome. The tumor may
rob the host of 50% of the available tryptophan. Its coexistence
with Crohn's disease has been reported (Wood et al., 1970).

Architecture of the small intestine is also disturbed in about
70% of the cases of generalized amyloidosis. Malabsorption syndrome
with steatorrhea appears to be relatively rare having been found in
only 5 to 6% of cases reported (Kyle and Bayrd, 1975).

Scleroderma may involve the small bowel and even the colon
resulting in dilatation and stasis leading to bacterial overgrowth.
About 50% of patients with small bowel involvement exhibit malab-
sorption with increased fecal fat and impaired D-xylose and B_{12}
absorption (Kahn et al., 1966).

Lymphatic Transport

The diseases that have been associated with abnormal lymphatic
transport of fat are primary lymphatic disease, Whipple's disease,
constrictive pericarditis and congestive heart disease. At first
lipids are removed from the G.I. lumen but do not pass from the
lymphatics into the circulation preventing continued uptake of
lipids. Steatorrhea appears with all its sequela.

ACTIVITY OF INTESTINAL MICROFLORA

Contaminated Small Bowel Syndrome (CSBS)

     The significance of the contaminated small bowel syndrome in
many diseases is becoming appreciated. The normal gut flora con-
sists of high numbers of anaerobes in a highly reducing medium in
the colon, and very low numbers of aerobic flora in the upper
tract (Gracey, 1979). This changes in many clinical situations
which are complicated by the overgrowth of bacteria. Anatomical
abnormalities of the gut and situations with abnormal gut motility
are frequently complicated by bacterial overgrowth as are many
postoperative states. These conditions may cause stasis resulting
in higher counts and shifts in kind of bacteria found in the upper
tract. A case report on CSBS as a complication of disordered in-
testinal motility due to amyloidosis was recently described
(Battle et al., 1979).

     A number of the diseases that can be classified in previous
categories are complicated by CSBS. In these diseases, the
bacterial overgrowth is probably due to the stasis as in Crohn's
disease, or to the increased concentration of nutrients that occur
in the tract. Solutes left in the gut lumen stimulate bacterial
growth. The microflora then compete with the host for essential
nutrients while producing products that alter the function of the
more distal portions of the tract. Unconjugated bile acids formed
from bacterial deconjugation are believed to alter water transport
(Teem and Phillips, 1972). The osmotic load can be increased by
the action of colon bacteria on carbohydrates yielding mono-
saccharides and organic anions. Water absorption is inhibited by
deoxycholate and hydroxy fatty acids formed by colon bacteria from
conjugated cholate and dietary fat (Soong et a., 1972). In CSBS,
B_{12} absorption may be compromised in that the microflora produce
inactive cyanocobalamin analogues from vitamin B_{12} which are ab-
sorbed, displace B_{12} from liver stores (Brandt et al., 1975) and
interfere with some binding assays (Kohlhouse et al., 1978);
inactive vitamin B_{12} analogues that are not absorbed or are poorly
absorbed are also formed in the gut where there is bacterial over-
growth and might interfere with absorption of vitamin B_{12} (Brandt
et al., 1977). Phillips (1972) has summarized the sequence of
events now believed to occur in diarrhea.

Other Activities of Microflora

     Not all activities of the microflora are detrimental to the
host. Vitamin K and folate are synthesized in the gut and are
available to the host. Bacterial metabolism of compounds secreted
into the gut allows excretion of unwanted compounds via the feces.

The metabolic activity of the flora and enteric cycling of urea, uric acid and creatinine has been investigated in detail. Efforts have been made to determine the quantity cycled, the factors which alter the quantities cycled and the products formed. An estimated 9 liters of water, 2 from the diet and 7 from intestinal secretions, are cycled through the G.I. tract per day (Phillips, 1972). Urea and creatinine are distributed in body water and thus, readily secreted into the G.I. tract. Interest in enteric cycling of these compounds is from two directions – reutilization of nitrogen, which would be expected to improve the state of protein nutrition, and elimination of metabolic products (metabolites can be trapped in the gut by use of sorbents). The clinical situations where this applies are in liver and kidney failure.

In hepatic coma, the object is to reduce the ammonia load to the liver, as 25% of the urea produced is enterically cycled and subject to conversion to NH_3 by gut bacterial urease. The removal of such a "toxin" could be of clinical significance. In uremia, the patient also cycles his body fluids through the G.I. tract. In this case, not only are the serum concentrations of those compounds normally excreted via the urine increased, but many of the products of bacterial metabolism are absorbed and are also retained.

Our studies on creatinine were prompted by the observation of Enger and Blegen (1964) that, as the serum concentration of creatinine increased, urinary excretion decreased. This decrease was not related to decreased muscle mass and suggested that creatinine was subject to enteric cycling similar to that of urea (Walser and Bodenlos, 1959) and uric acid (Sorenson, 1959).

In a series of experiments, we demonstrated:

1. Induction of "creatininase" in the intestinal flora of the rat fed creatinine but not creatine (Jones and Burnett, 1972).

2. Enteric cycling of up to 60% or 700 mg of creatinine formed per day in uremics (Jones and Burnett, 1974).

3. That the proposed pathways for creatinine degradation by gut bacteria shown in Fig. 1 are followed in uremic patients (Jones and Burnett, 1975).

The uremic patient has bacterial overgrowth in his small bowel (Simenhoff et al., 1976) and thus forms these and other compounds in the G.I. tract in quantities sufficient to permit absorption from the gut.
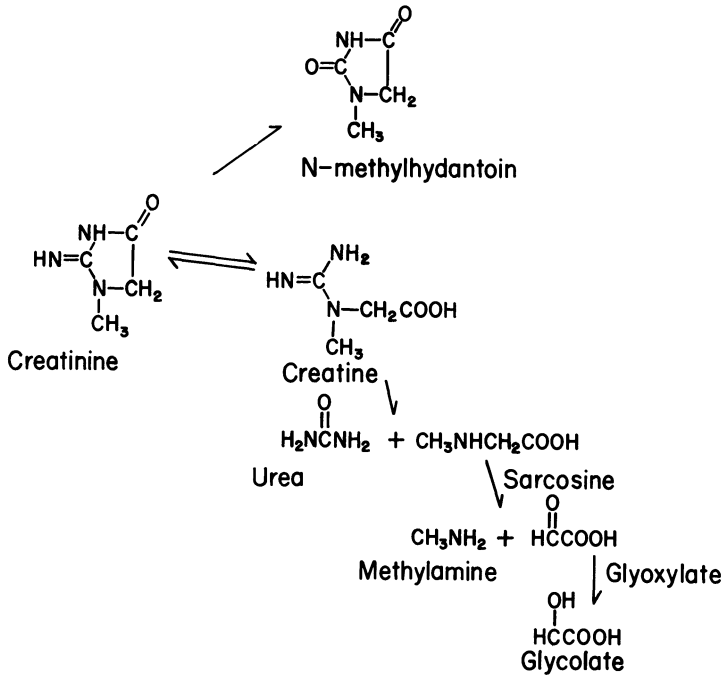
Fig. 1. Metabolism of Creatinine by the Microflora of the Intestine in Uremia. (Reprinted from Kidney International, Jones and Burnett (1975), with permission.)

    The quantitative significance of enteric cycling has been determined by direct measurement of urea, creatinine and uric acid. The biochemical significance waits on the elucidation of the fate of the metabolites and of any affects the metabolites have on the host.

SUMMARY

    Efficient digestion and absorption of ingested foodstuffs requires an intact, functional gastrointestional tract. Interferences with digestive processes may cause loss of specific dietary nutrients and also loss of nutrients secreted into the tract. The diseases associated with malabsorption may be classified according to:

    Abnormalities of the intraluminal phase
    Abnormalities of mucosal cell transport
    Abnormalities of intestinal lymphatic transport.

Impaired intestinal absorption of nutrients which may present as the malabsorption syndrome as a result of interaction of dietary components or by deficiency of specific nutrients, e.g., vitamin D, are reviewed. Effects of contaminated bowel in many disease states is emphasized.

Diagnostic approaches are based on the biochemistry of digestion and transport of nutrients. Consideration of the processes involved in malabsorption allows better interpretation of diagnostic test and understanding of the effects of these disease processes on other laboratory values.

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AMINO ACIDS IN HEALTH AND DISEASE

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INTRODUCTION

The proteins of the human body are in a constant state of
flux with some proteins being synthesized and catabolized con-
tinually. Dietary proteins and tissue protein breakdown products
provide a pool of amino acids which provide the building blocks
for formation of new protein for growth and for rebuilding tissue
protein. Essential amino acids are those not synthesized by man
or not produced sufficiently rapidly enough to support this acti-
vity. Therefore, man requires a source of nitrogen and essential
amino acids to support growth, protein synthesis and biosynthesis
of important nitrogenous metabolites. The differences in value of
food proteins are due to variations in amino acid pattern of each
protein. Since the body can form only some of the amino acids,
nine "essential" ones must be supplied in the diet (Table I)
(McLaughlan et al., 1963, Schelling, 1975). Protein normally is
not absorbed by the human intestine, but its hydrolysis products
peptides and free amino acids are readily absorbed by the small
intestine and transported via the portal blood. A diet low in
protein or food protein which is deficient in one or more essen-
tial amino acids will restrict protein synthesis. Nonessential
amino acids are generally metabolized to pyruvate which leads to
gluconeogensis. Therefore, in times of great need (starvation)
body proteins can provide energy via glucose. The problem here is
that the conversion of protein to glucose is only about 50% effi-
cient (Bessman, 1975) so that 100 g of glucose will require 200 g
of protein or the equivalent of 1 kg of lean body weight. Most of
the essential amino acids are converted to acetyl CoA and their
nitrogen as well as that of the amino acids converted to glucose
is lost to the body primarily as ammonia.

Table I.   Essential Amino Acid Requirements in Healthy
           Human Infants

Amino Acid	Mean (mg/kg/day)
Histidine	34
Isoleucine	126
Leucine	150
Lysine	103
Methionine	45
Phenylalanine	90
Threonine	87
Tryptophan	22
Valine	105

(From Holt et al., 1960)

PROTEIN REQUIREMENTS

A well balanced diet containing 8-10% protein from various
sources will satisfy the needs of the adult for nitrogen and
essential amino acids as well as provide sufficient caloric con-
tent to prevent utilization of amino acids for energy (National
Academy of Sciences, 1968).

Infants up to a year of age require 1.8-2.2 g of protein/kg/
day. After a year of age, the gain in body weight is about 3%
nitrogen or 18% protein and the requirements to maintain this in-
crement plus the obligatory loss ranges from 2.1 g to 2.3 g of
protein/kg/day. The gain in body protein per day then is equal to
the daily weight gain multiplied by 0.18. Growth increases the
protein requirements enormously, from 2 g at birth to 60 g for
adolescent girls, and 80 g for adolescent boys (Wehr and Lewis,
1966, National Academy of Sciences, 1968). In adulthood, about
1 g protein/kg/day is sufficient to maintain body weight (Holt
et al., 1960). In healthy persons heavy muscular activity does
not change the need for protein unless hypertrophy of muscle is in
progress or heavy sweat losses are sustained. Pregnancy requires
increases of 0.5 g of protein/day in the first trimester and up to
5.4 g/day in the last. Lactation increases the protein require-
ment 16 g/day. Menstruation causes very little loss of nitrogen
(0.3 mg protein/day/kg). The normal stress encountered in daily
life does not appreciably change the need for protein but the
stress of serious infection, burns, or anemia temporarily raises
the protein needs of the individual. Severe food restrictions,
acquired and genetic disease effects will be discussed later in
this work.

FACTORS AFFECTING PLASMA AMINO ACID CONCENTRATIONS

   Three things must be considered when examining amino acid
concentrations in the human; methodology, intermediary metabolism
and genetics.

Methodology

   Analytical methods for amino acids go back to the beginning
of the 20th century, when Garrod (1908) introduced the concept of
inborn errors of metabolism. At that time only two disorders in-
volving amino acids were known (alcaptonuria and cystinuria) and
were diagnosed by simple tests applied to the patients urine.
Two more disorders were discovered by similar methods, phenyl-
ketonuria and tyrosinosis (Medes type) in the next 30 years. The
advent of partition chromatography coupled with the ninhydrin
strain in the 40's spawned the discovery of some 50 "ninhydrin-
positive" traits (Dent, 1946). The application of gas chromato-
graphy and mass spectrometry in the 60's helped reveal "ninhydrin-
negative" traits of amino acid metabolism (Horning and Horning,
1971). The choice of the several methods for examining amino acid
metabolism in humans depends upon the goal of the analyst; whether
qualitative or quantitative, specific or comprehensive, manual or
automated. Qualitative assay of amino acid mixtures may be
achieved by chemical (Hill et al., 1965), Hochella, 1967), micro-
biological (Guthrie and Susi, 1963), chromatographic (Efron et al.,
1964, Levy, 1968, Raine et al., 1972, Tocci, 1967, Jackson et al.,
1968) and electrophoretic (Efron, 1959, 1968) techniques. Indivi-
dual amino acids are generally estimated by fluorometric methods
(Waalkes and Udenfriend, 1957, McCamen and Robins, 1962). Liquid
elution chromatography utilizing the ninhydrin reaction has been
the standard for quantitative analysis of complex amino acid mix-
tures (Moore and Stein, 1954) though recently gas chromatography
(Gehrke et al., 1968) and liquid-elution chromatography utilizing
fluorescence are becoming popular (Benson and Hare, 1975).

   All methods of analyses of the amino acids have pitfalls of
which one must be aware in order to recognize an abnormal finding
when it is encountered: venipuncture will lower the taurine and
glutamic acid concentration for up to one hour (Rouser et al.,
1962); plasma is preferred to serum since it can be processed
faster (serum may change its amino acid composition while standing
at room temperature during clot retraction), the use of excess
heparin as an anti-coagulant may cause hemolysis which will alter
the amino acid pattern, and impurities in some lots of EDTA can
produce spurious peaks in the liquid elution chromatogram (Perry
and Hansen, 1969); plasma specimens can be contaminated by plate-
lets and leukocytes which contain large amounts of taurine and
dicarboxylic acid (Rouser et al., 1962; Soupart, 1962) hemolysis
causes the appearance of glutathione, increases ornithine, and

reduces arginine and possibly cystine (Winter and Christensen, 1964; Levy and Barkin, 1971).

Immediate deproteinization is important to avoid losses of disulfide amino acids which bind to plasma proteins at room temperature or in the refrigerator (Moore and Stein, 1954). Deproteinization may be accomplished with 3% sulfosalycylic acid (Gerritson et al., 1965) or picric acid (Moore and Stein, 1954). After deproteinization, storage for long periods of time at $-20^{\circ}C$ raises tryptophan, glutamine and aspartic acid concentrations (Scriver and Rosenberg, 1973). Other factors which may affect amino acid concentrations are continued fasting and food intake (Weller et al., 1969; Adibi, 1970), while physical activity and choice of arm for venipuncture have no influence (Armstrong and Stave, 1973).

Intermediary Metabolism

After birth, the amino acid concentrations of plasma fall in the first few hours of life (Dickenson et al., 1965; Linblad and Baldeston, 1969). Placental insufficiency can also lower amino acid concentrations in the newborn. The maternal plasma amino acid concentrations are reduced significantly in the first half of pregnancy, especially citrulline, ornithine, and arginine. The concentrations rise toward normal at term (Reid et al., 1971). Maternal protein deprivation can be devastating to the developing fetus (Naeye et al., 1973; Mestyan et al., 1969; Bessman, 1972). There is circadian periodicity to the concentrations of plasma amino acids (Wurtman et al., 1968; Feigin et al., 1968) which is not great, but can effect the interpretation of plasma amino acid concentrations in research protocols where repeated sampling of blood for plasma amino acids may be necessary (Feigin and Haymond, 1970), or in the identification of heterozygotes for phenylketonuria (Rosenblatt and Scriver, 1968). Sex-related differences are minor (Dirren et al., 1975). Amino acid deficiency in man is usually a function of protein malnutrition. The use of synthetic or modified diets has added to this classification because of the attendant hazard of deficiencies involving the essential amino acids. These problems are particularly severe in the neonate, since at birth skeletal muscle comprises only 20-25% of the birth weight and although amino acids can be metabolized to provide calories, neither glucose nor fat can act as precursors for essential amino acids. Special care must be taken in the interpretation of amino acid results from infants on amino acid supplementation, given either orally or intravenously (Logan et al., 1974; Brans et al., 1974; Ghadimi et al., 1971; Ghadimi, 1973; Abitol et al., 1975; Heird and Winters, 1975) and adults given intravenous amino acid solutions after surgical operations (Tweedle et al., 1973; Furst et al., 1978). Starvation results in cyclic changes in the amino acid concentrations of plasma that reflect the changing

amino acid pools and the splanchnic handling of amino acids (Felig
et al., 1969; Adibi et al., 1970; Cahill, 1970). Branched chain
and other amino acids are the first to leave the muscle and enter
the plasma as a result of the loss of insulin control in muscle
pools. Then, the concentrations of alanine and other glycogenic
amino acids begin to fall in the plasma because the liver utilizes
them for gluconeogenesis. Later as starvation progresses alanine
remains low and the drain of protein is diminished as the brain
adapts to operating on ketone bodies instead of glucose (Owen
et al., 1967). When this occurs the kidney assumes the larger
role of gluconeogenesis and ammoniagenesis. It utilizes glycine,
alanine and proline to manufacture glucose and glutamine for
ammoniagenesis. Plasma glycine concentration steadily rises
unexplainably as alanine and the branched chain amino acids
steadily decline (Cahill, 1970).

    In protein-calorie malnutrition, which differs from starva-
tion, the concentrations of proline, serine, glycine, an phenyla-
line rise in the plasma while leucine, iso-leucine, valine, trypto-
phan, methionine, threonine, arginine and citrulline are in rela-
tive deficiency (Holt et al., 1963). There are large oscillations
of the plasma amino acid concentrations during the early stages
(Heard et al., 1969; Whitehead, 1967) which make treatment diffi-
cult, probably due to the loss of tissue pools for storage and
enzymes for metabolism of the amino acids, as well as intestinal
changes which compromise the availability of dietary nitrogen.
Maternal malnutrition causes changes in the amino acid patterns
of the fetus which persist after birth (Mestyan et al., 1969).

    Several attempts to determine the extent of protein and
calorie deprivation have been based on the aminogram (quantitative
amino acid analysis results); in marasmus (Heard et al., 1969;
Arroyove et al., 1962; Whitehead, 1967; Arroyove and Bowering,
1968) malnutrition (Baertle et al., 1964; Westall et al., 1958),
and kwasiorkor (Whitehead and Dean, 1964; Rutishauser and
Whitehead, 1969; Holt et al., 1963). However, the aminogram is
of little use in diagnosing the state of the protein economy in
the normal human, since the free amino acids constitute only 0.1%
of the total body protein. Normal variations of the amino acids
are often as much as 200-300% as compared with approximately 10%
variation in blood electrolytes. Obesity also alters plasma amino
acid concentrations in non-diabetic people (Felig et al., 1969).
Glycine is depressed while tyrosine, phenylalanine and the
branched-chain amino acids are significantly elevated. This is
probably a reflection of the ineffectiveness of insulin to control
amino acid transportation into and out of skeletal muscle.
Diabetes in its various manifestations also alters the aminogram
probably by shifting of amino acids from muscle to liver (Felig
et al., 1970 and Aoki et al., 1975). Disorders affecting the liver
and kidney (McGale et al., 1972; Nyhan and Tocci, 1966;

Hilty et al., 1974; Furst et al., 1978) as well as endocrine and
muscular disorders (Felts, 1971; Kisser, 1974) very often disturb
the homeostasis of amino acid metabolism.

Genetics

     For clinical purposes the most important application of amino
acid analyses is the determination of free amino acids in physio-
logical fluid and tissue as a key to the diagnosis of the more
than 50 inborn errors of amino acid metabolism. These diseases
are relatively rare and are seen mainly in the newborn. Primary
aminoacidopathies are caused by the lack of an enzyme or enzyme
activity for intermediary metabolism and are usually of autosomal
recessive inheritance. They affect only one or a few amino acids.
The clinical signs and symptoms of genetic amino acid disorders
are varied and diffuse so that the differential diagnosis of amino
acid disorders is extensive. It is important to recognize these
disorders since many of them are treatable if diagnosed early.
Most of the inborn errors of amino acid metabolism cause severe
mental retardation, sickness and early death in the untreated
state. For this reason, there are large screening programs for
detecting them in the neonate (Jackson et al., 1968; Levy et al.,
1968). Even when the opportunity is missed for early diagnosis
the correct diagnosis of a genetically determined aminoacidopathy
is of great importance for genetic counseling of the family, the
early diagnosis of abnormalities of newborns in the family, and
the determination of the carrier state in siblings. There are
genetic disorders involving amino acid metabolism for which treat-
ment may be vitamin therapy, rather than restriction or addition
of protein or specific amino acids (Sherwood and Paris, 1964).
Final confirmation of many of those disorders is often difficult
since a liver biopsy may be needed (Scriver and Rosenberg, 1973)
or may be impossible since all the enzymes are not yet known.

     High urinary excretion of amino acids can occur in the absence
of aminoacidemia due to a defect in the renal tubular reabsorption
mechanism. Some of these disorders are also characterized by a
disturbance of intestinal reabsorption of certain amino acids
(Segal, 1976; Scriver, 1969).

SUMMARY

     In summary, the plasma amino acid concentration in human
beings are amazingly constant in healthy adults. On the other
hand, there are methodological problems, variations between indivi-
duals, small changes due to acquired disease or due to fad diets
or very large changes due to genetic disorders or amino acid
supplementation of which one must be aware to interpret correctly
the results of amino acid analyses and intelligently apply this
information in the treatment of patients.

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AMINOGRAMS IN VARIOUS TYPES OF LIVER DISEASE

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INTRODUCTION

The concentration of amino acids in the serum is significantly
altered in various liver diseases (Fisher, 1975; Iber, 1957;
Mellinkoff, 1955; Ning, 1967; Rosen, 1977). While there is consi-
derable information about the amino acid concentrations in cirrho-
sis and hepatic encephalopathy, there is only limited information
about the serum amino acid concentration in liver diseases other

than cirrhosis and hepatic encephalopathy (Mellinkoff, 1955; Ning, 1967). Accordingly, we studied the amino acid concentrations in the serum of patients with different liver diseases for the following purposes:

> (a) To recognize consistent derangements, if any, of the amino acid concentrations in different types of liver disease.

> (b) To make a comparative study of the amino acid concentrations in various liver diseases.

> (c) To define the diagnostic and prognostic significance of alterations in amino acid concentrations by correlating these changes with the severity of the histological findings.

MATERIALS

Sera from 68 fasting patients with different forms of liver diseases and 9 healthy hospital workers were studied. The various types of liver disease and the number of patients in each group are described in Table I. The clinical diagnosis of the type of liver disease in all patients was confirmed by the histological examination of liver tissue obtained by needle biopsy, except in the patients with coma due to hepatic or non-hepatic causes. All patients in coma were in stage IV when the blood was drawn for amino acid determination. The five patients with coma of non-hepatic origin had no liver disease by history, physical examination or routine biochemical tests of liver function. The patients with hepatic coma had clinical evidence of hepatic disease-ascites, jaundice, asterixis, neurological findings and gross abnormalities of liver function.

METHODS

Venous blood samples were collected in dry tubes. The serum was immediately separated by centrifugation, frozen and stored at $-18^{\circ}C$ until used. One ml of this serum was deproteinized by the addition of 0.1 ml of 50% (w/v) sulfosalicylic acid. The supernatant was recentrifuged (Beckman Microcentrifuge) and 25 μl of 4% (w/v) lithium hydroxide was added to 400 μl of the supernatant to adjust to a proper pH. One hundred μl of this mixture was injected onto a 460 x 6 mm column with Beckman's spherical resin, Type W-3P (Beckman Instrument Model 119 CL Amino Acid Analyzer) together with 100 μl of α-amino, β-guanidino, propionic acid hydrochloride. The final concentration of various amino acids was calculated and integrated by the automated Beckman Model 126 and reported in μmoles/liter.

The mean value of each amino acid of each disease group was compared to the mean normal value of that amino acid by discriminatory analysis. The following chemical analyses were used as part of liver function: Total protein, albumin, globulin, gammaglobulin, alkaline phosphatase, cholesterol, total and direct bilirubin, aspartate aminotransferase, alanine aminotransferase, isocitric dehydrogenase and lactate dehydrogenase.

Table I. Numbers of Patients in the Various Groups Studied

GROUP	NUMBER
Normal	9
Hepatic Coma	10
Non-Hepatic Coma	5
Acute Viral Hepatitis	11
Chronic Active Hepatitis	6
Fatty Liver	7
Fatty Cirrhosis	9
Advanced Cirrhosis	20
TOTAL	77

RESULTS

Normal

The results of the amino acid determinations in normal individuals are presented in Table II.

Fatty Liver

Methionine, phenylalanine and serine were the only amino acids whose concentration increased significantly ($p < 0.05$). All other amino acids showed no significant change (Table III).

Fatty Cirrhosis

Patients with moderate cirrhosis associated with mild to moderate fatty changes showed a significant increase ($p < 0.05$),

Table II. Normal Fasting Concentrations of Amino Acids
           in Serum

AMINO ACID	MEAN (μMOLES/LITER)	STD. DEV.	RANGE
Phosphoserine	5.2	0.8	3.6- 6.3
Taurine	106.3	25.3	69.1-147.4
Aspartic Acid	24.9	3.1	19.1- 28.1
Hydroxyproline	13.9	8.1	8.7- 31.4
Threonine	107.0	21.2	77.7-135.0
Serine	111.2	21.0	68.2-136.0
Asparagine	54.4	13.1	36.4- 72.5
Glutamic Acid	55.6	13.8	28.0- 76.8
Glutamine	494.3	113.6	317.0-645.0
Proline	196.5	69.9	92.1-301.0
Glycine	200.7	30.3	146.0-228.0
Alanine	390.4	92.3	300.7-518.0
Citrulline	17.0	14.4	5.4- 51.3
α-Amino-n-Butyric Acid	12.7	6.9	7.1- 26.3
Valine	166.9	33.0	122.4-212.0
1/2 Cystine	65.6	15.4	47.1- 94.0
Methionine	13.0	6.5	5.2- 17.0
Cystathionine	0.83	0.24	0.50-1.10
Isoleucine	45.8	9.9	30.2- 58.0
Leucine	100.9	16.7	77.0-125.0
Tyrosine	48.1	6.5	35.2- 54.1
Phenylalanine	49.4	6.5	39.7- 57.6
Tryptophan	36.9	7.2	27.5- 50.5
Ammonia	372.1	41.2	319.8-426.0
Ornithine	80.7	16.7	57.6-117.0
Lysine	150.5	21.1	118.4-181.1
Histidine	65.0	8.9	53.2- 76.2
Arginine	66.6	12.8	47.7- 70.9

Table III. Differences in Serum Amino Acids in Fatty
Liver and Liver Cirrhosis

	μMOLES/LITER			
	NORMAL	FATTY LIVER	FATTY CIRRHOSIS	ADVANCED CIRRHOSIS
Aspartic Acid	24.9	45.6	45.6(+)	42.3(+)
Serine	111.2	145.1(+)	155.1(+)	152.7(+)
Asparagine	54.4	72.1	70.7	99.3(+)
Glutamic Acid	55.6	230.5	357.0	270.3(+)
Glutamine	494.3	374.6	154.8(-)	197.7(-)
1/2 Cystine	65.6	31.0	7.6(-)	25.4(-)
Methionine	13.0	35.6(+)	21.9(+)	43.5(+)
Isoleucine	45.8	65.6	59.2(+)	57.3
Tyrosine	48.1	62.9	65.9(+)	102.3(+)
Phenylalanine	49.4	69.0(+)	73.0(+)	82.4(+)
Ammonia	372.1	385.2	422.7	453.6(+)
Arginine	66.6	93.1	91.4	108.4(+)

(+) and (-) indicate significant increase and decrease,
respectively, from normal ($p < 0.05$).

in aspartic acid, serine, methionine, and in the aromatic amino
acids, tyrosine and phenylalanine concentrations. The only
branched chain amino acid that showed an increased concentration
was isoleucine.

The concentrations of glutamine and half-cystine were markedly
decreased. All other amino acids studied showed no significant
changes from the normal (Table III).

Advanced Cirrhosis

     Phenylalanine and tyrosine concentrations were significantly
increased (p <0.05).

     The concentrations of methionine, serine, asparagine, glutamic
acid, aspartic acid, arginine and ammonia were increased to a
lesser degree. Glutamine and half-cystine levels were signifi-
cantly decreased. The other amino acids showed no significant
changes (Table III).

Hepatitic Coma

     The concentrations of phenylalanine, methionine, glutamic
acid and tyrosine showed the most marked elevation. The concentra-
tions of serine, arginine and ammonia were significantly increased
(p <0.05). The concentrations of leucine and valine were markedly
decreased while those of isoleucine and tryptophan remained
unchanged.

     The other amino acids studied showed no statistically signi-
ficant changes (Table IV).

Non-Hepatic Coma

     In non-hepatic coma the concentrations of phenylalanine,
glycine, serine, asparagine and glutamic acid were significantly
increased, while those of tyrosine, tryptophan, valine, leucine,
isoleucine and others showed no significant change (Table IV).

Acute Viral Hepatitis

     Phenylalanine, tyrosine, methionine, taurine, threonine,
serine, lysine and arginine were significantly elevated. The con-
centration of tryptophan did not increase significantly.

     The concentrations of the branched chain amino acids leucine
and isoleucine were increased while valine was not significantly
altered.

     There was marked decrease in the concentration of half-cystine
(Table V).

Chronic Active Hepatitis

     Patients with chronic active hepatitis showed a mild increase
in the concentration of many amino acids, though in only a few of
them was the increase significant. The concentrations of tyrosine,
phenylalanine, methionine and glutamic acid were markedly increased

Table IV. Differences in Serum Amino Acids Between
Hepatic Coma and Non-Hepatic Coma

	HEPATIC COMA		NON-HEPATIC COMA	
	μMOLES/LITER	/NORMAL	μMOLES/LITER	/NORMAL
Phosphoserine	9.9	+1.895	5.2	+0.989
Hydroxyproline	6.6	-0.476	14.5	-1.046
Serine	157.6	+1.417	141.3	+1.270
Asparagine	73.6	-1.351	81.5	-1.497
Glutamic Acid	196.0	+3.523	145.1	+2.608
Proline	210.4	+1.071	149.8	+0.762
Glycine	317.7	+1.583	270.6	+1.348
Citrulline	21.5	+0.722	15.4	+0.858
Valine	101.5	-0.608	172.1	-1.031
Methionine	61.3	+4.708	16.8	+1.292
Leucine	72.2	-0.716	103.5	-1.026
Tyrosine	70.3	+1.460	46.1	+0.973
Phenylalanine	102.2	+2.068	73.2	+1.482
Ammonia	494.2	+1.328	326.2	+0.877
Ornithine	82.3	-1.020	100.6	-1.247
Arginine	98.3	+1.476	62.5	+0.938
T = 3.291, p <0.01		+16.580 ± 6.091		+6.495 ± 3.671

(p <0.05). Leucine, valine, threonine, asparagine and lysine were
only slightly (but not significantly) increased. The only amino
acid that was markedly decreased was that of half-cystine (Table V).

Table V. Differences in Serum Amino Acids in Acute
          and Chronic Active Hepatitis

	μMOLES/LITER		
	NORMAL	ACUTE HEPATITIS	CHRONIC ACTIVE HEPATITIS
Threonine	106.7	217.3(+)	138.8(+)
Serine	111.2	206.2(+)	156.8
Asparagine	54.4	104.5	83.0
Glutamic Acid	55.6	265.8	286.6(+)
Valine	166.9	204.1	218.6(+)
1/2 Cystine	65.6	18.4(-)	1.3(-)
Methionine	13.0	51.5(+)	32.6(+)
Isoleucine	45.8	68.8(+)	74.8
Leucine	100.9	138.1(+)	147.0(+)
Tyrosine	48.1	92.5	76.8(+)
Phenylalanine	49.4	93.4(+)	95.2(+)
Ammonia	372.1	448.9(+)	430.4
Lysine	150.5	217.0(+)	208.6(+)
Arginine	66.6	102.5(+)	82.7

(+) and (-) indicate significant increase and decrease,
respectively, from normal (p <0.05).

Fatty Liver and Fatty Cirrhosis

Glutamine, half-cystine and methionine were considerably more
decreased in fatty cirrhosis than in fatty liver.

When comparing phosphoserine, taurine, methionine, cystathio-
nine and lysine between these two groups, statistically significant
differences were seen (Table VI).

Table VI. Elevations in Serum Amino Acids Above Normal
in Fatty Liver and Fatty Cirrhosis

	FATTY LIVER		FATTY CIRRHOSIS	
	μMOLES/LITER	/NORMAL	μMOLES/LITER	/NORMAL
Phosphoserine	7.3	+1.393	4.7	+0.895
Taurine	128.2	+1.206	111.1	+1.045
Methionine	35.6	+2.739	21.9	+1.683
Cystathionine	2.0	+2.424	1.7	+2.024
Lysine	176.4	+1.172	163.1	+1.084
Normal* = 5.000 ± 0.778		+8.934 ± 2.614 (T = 3.949, p <0.01)		+6.731 ± 1.626 (T = 2.296, p <0.05)

*The algebraic sum of this discriminant in the normal population
in which the mean value of each amino acid is assigned the
value 1.00.

Fatty Cirrhosis and Advanced Cirrhosis

The concentrations of tyrosine, methionine, cystathionine,
arginine, half-cystine, proline and asparagine were higher in
advanced cirrhosis than in fatty cirrhosis. In fatty cirrhosis,
taurine, glutamic acid and lysine were more elevated than in ad-
vanced cirrhosis (Table VII).

Hepatic and Non-Hepatic Coma

In patients with hepatic coma, methionine was increased to
more than four times normal, while it was only slightly elevated in
non-hepatic coma.

Tyrosine, phenylalanine and glutamic acid were also elevated
much more in hepatic than in non-hepatic coma.

Leucine, valine and hydroxyproline were significantly lower
in hepatic than in non-hepatic coma (Table IV).

Table VII. Differences in Serum Amino Acids Between
           Fatty Cirrhosis and Advanced Cirrhosis

	FATTY CIRRHOSIS		ADVANCED CIRRHOSIS	
	μMOLES/LITER	/NORMAL	μMOLES/LITER	/NORMAL
Taurine	111.1	+1.045	90.1	+0.848
Threonine	109.8	-1.026	130.5	-1.220
Asparagine	70.7	-1.299	99.3	-1.824
Glutamic Acid	357.0	+6.417	270.3	+4.859
Proline	148.2	-0.754	203.1	-1.034
1/2 Cystine	7.6	-0.116	25.4	-0.387
Methionine	21.9	-1.683	43.5	-3.342
Cystathionine	1.7	-2.024	4.6	-5.576
Tyrosine	65.9	-1.370	102.3	-2.126
Lysine	163.1	+1.084	137.7	+0.915
Histidine	60.3	-0.928	68.0	-1.047
Arginine	91.4	-1.372	108.4	-1.628
T* = 2.842, p <.01		-2.026 ± 3.632		-11.562 ± 9.714

*The T statistic refers to the difference between the mean
discriminant values of the two disease groups.

Acute Viral Hepatitis and Chronic Active Hepatitis

    Most of the amino acid levels tended to be significantly
higher than normal in both of these conditions. No statistically
significant differences were found between these two groups
(Table VIII).

Table VIII. Differences in Serum Amino Acids Between
Acute Viral Hepatitis and Chronic Active
Hepatitis

	ACUTE VIRAL HEPATITIS		CHRONIC ACTIVE HEPATITIS	
	μMOLES/LITER	/NORMAL	μMOLES/LITER	/NORMAL
Taurine	160.5	+1.510	111.0	+1.044
Hydroxyproline	7.7	−0.551	13.1	−0.942
Threonine	217.3	+2.031	138.8	+1.297
Serine	206.2	+1.854	156.8	+1.410
Asparagine	104.5	+1.920	83.0	+1.526
Glutamine	384.6	+0.778	305.0	+0.617
Glycine	304.8	+1.519	270.4	+1.347
1/2 Cystine	18.4	+0.280	1.3	+0.020
Methionine	51.5	+3.958	32.6	+2.504
Tyrosine	92.5	+1.922	76.8	+1.596
Tryptophan	57.4	+1.554	40.8	+1.105
Arginine	102.5	+1.539	82.7	+1.241
T* = 1.677, N.S.		+18.314 ±6.344		+12.765 ± 3.159

*The T statistic refers to the difference between the mean
discriminant values of the two disease groups.

DISCUSSION

     The concentrations of serum amino acids are not only altered
in various liver diseases but the pattern of change also seems to
vary from one pathological group to another.

     The patients with hepatic coma showed consistent increase in
methionine, phenylalanine and tyrosine. Tryptophan was unaltered.
Branched chain amino acids (BCAA), leucine and valine were de-
creased while isoleucine was unaffected. Similar changes accom-
panied by decreased isoleucine concentrations have been shown in
other studies (Fisher, 1976; Iber, 1957; Rosen, 1977; Silverman,
1968; Steigmann, 1970). Taurine concentrations have been reported
to be increased in hepatic encephalopathy (HE) (Rossi-Fanelli,
1976).

     It has been proposed that patients with hepatic coma have
decreased BCAA because of hyperinsulinemia which enhances the
uptake of BCAA by the muscle and adipose tissue (Munro, 1975;
Soeters, 1976). The aromatic amino acids (AAA) are elevated be-
cause of the failure of the liver to metabolize them as shown by
Munro (1975) and Soeters (1976). BCAA compete with AAA for trans-
port across the blood brain barrier so that a decrease in the
BCAA would facilitate the passage of AAA into the cerebrospinal
fluid (James, 1978). Excesses of AAA will inhibit the synthesis of
catecholamines (Wurtman, 1971). Octopamine is produced as a
result from phenylalanine and tyrosine as shown by Meredith (1979).
Excess of tryptophan leads to increased production of serotonin
(Fernstrom, 1972). Increased methionine levels may lead to in-
creased methanethiol which has a synergistic effect with ammonia
(Condon, 1970; Merino, 1975). These end products may be related
to the pathogenesis of hepatic coma.

     In patients with advanced cirrhosis but without coma, the
concentrations of BCAA were near normal, though phenylalanine,
tyrosine and methionine were increased. It would therefore appear
that patients with advanced cirrhosis would be triggered into HE
by the decrease in BCAA and conversely, the patients with hepatic
coma would improve by increase in their BCAA. This has indeed
been shown by Fischer et al. (1976).

     The patients with coma of non-hepatic etiology showed an
increase in phenylalanine while other AAA and BCAA were near
normal. The pathogenesis of non-hepatic coma may be related to
factors other than the ratio of AAA to BCAA.

     The amino acid pattern seems to go from bad to worse as his-
tological changes progress from fatty liver to advanced cirrhosis
and coma. The change is both qualitative and quantitative. In

fatty liver only three amino acids were altered from the normal
while in fatty cirrhosis and advanced cirrhosis 8 and 12 amino
acids were affected. Among the altered amino acids the concentra-
tion was most markedly different from the normal in the most
severe liver diseases, i.e., hepatic coma and advanced cirrhosis.
Similarly, the quantitative changes were the least in those with
less severe disease, i.e., fatty liver and fatty cirrhosis.

The elevation of amino acid concentrations such as methionine,
tyrosine, asparagine, threonine and tryptophan is marked in acute
viral hepatitis while it is less so in chronic active hepatitis.
This elevation may be mainly due to release of amino acids from
the necrosed hepatocytes, though muscle catabolism may contribute
to a small extent (Rosen, 1977). Tyrosine concentrations have
been shown to correlate well with serum aspartate aminotransferase
activities and it has been suggested that AAA concentrations,
especially that of tyrosine, can be used to predict the extent of
hepatic necrosis (Rosen, 1977).

The AAA are markedly elevated in patients with coma due to
"fulminant hepatitis" while BCAA are near normal, thereby altering
the ratio of AAA to BCAA (Rosen, 1977). This alteration of ratio
may be related to causation of coma in fulminant hepatitis. In
our patients with acute viral hepatitis, both BCAA and AAA were
elevated, keeping the ratio near normal. Thus, it may be that
the concentrations of BCAA may bear some prognostic significance
and will determine the outcome of a case of viral hepatitis
(Dubin, 1979).

SUMMARY

Patients with advance cirrhosis showed significantly increased
phenylalanine and tyrosine, while methionine, serine, asparagine,
glutamic acid, aspartic acid, arginine and ammonia were increased
to a lesser degree, and glutamine and half-cystine levels were
significantly decreased. In hepatic coma phenylalanine, methio-
nine, glutamic acid and tyrosine showed the most marked elevation,
arginine and ammonia were significantly increased, and leucine
and valine were markedly decreased. In contrast, tyrosine, trypto-
phan, valine, leucine, isoleucine showed no significant change in
non-hepatic coma.

The alteration in the amino acid pattern, both qualitatively
and quantitatively correlates with the etiology and pathology of
liver disease. Different patterns of change can be seen in dif-
ferent liver diseases. It may be possible to predict the severity
and prognosis of liver disease by the changes seen in amino acids.
Therapy may accordingly be modified to correct the imbalance of
amino acids.

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THE ROLE OF ERYTHROCYTE AMINO ACIDS

IN ENERGY METABOLISM

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INTRODUCTION

    Although there is much information regarding free amino acids
in plasma, the amino acids of erythrocytes have been little studied
(Felig, 1973; Felig et al., 1970; Levy and Barkin, 1971; McMenamy
et al., 1960). However, accurate comparisons of the amino acid
concentrations of plasma and erythrocytes can result in a better
understanding of the relative role of these amino acid pools in
energy metabolism.

Amino Acid Content of Erythrocytes and Plasma

    The concentrations of amino acids in erythrocytes are quite
different from their concentrations in plasma (Table 1). While
many amino acids do not exhibit plasma: erythrocyte ratios dif-
ferent from 1.0, aspartate, glutamate, serine and glycine are
present in erythrocytes at higher concentrations than in serum
(McMenamy et al., 1960). On the other hand, at least in humans,
arginine is more concentrated in plasma than in erythrocytes (Levy
and Barkin, 1971). The presence of essentially equal concentra-
tions of many amino acids in plasma and erythrocytes, even in
metabolic disorders resulting in elevated concentrations, indicates
that amino acid transport is either passive or that the transport
systems have very high saturation points (Levy and Barkin, 1971).
It has become increasingly clear that the erythrocytes do not
passively reflect all amino acid concentrations. However, the
functional role of erythrocyte amino acids remains unclear, parti-
cularly since mature red blood cells do not synthesize protein.
This is reflected in the fact that amino acid concentrations in
reticulocytes, which synthesize protein, are quite different from

Table 1.   Amino Acid Concentrations in Plasma and
           Erythrocytes

	Concentration (µmole/L)	
	Plasma	Erythrocytes
Essential Amino Acids		
Isoleucine	49 ± 9	43 ± 8
Leucine	114 ± 9	100 ± 5
Lysine	164 ± 11	143 ± 18
Methionine	24 ± 1	11 ± 3
Phenylalanine	60 ± 7	61 ± 16
Threonine	134 ± 34	154 ± 55
Tryptophan	---	---
Nonessential Amino Acids		
Alanine	258 ± 34	250 ± 31
Arginine	55 ± 12	25 ± 15
Aspartate	0	264 ± 59
Citrulline	26 ± 14	25 ± 8
Cystine	73 ± 16	7 ± 8
Glutamate	56 ± 20	220 ± 49
Glutamine	283 ± 79	201 ± 38
Glycine	192 ± 21	264 ± 25
Histidine	117 ± 17	115 ± 8
Ornithine	129 ± 23	149 ± 2.8
Serine	171 ± 44	265 ± 39
Tyrosine	75 ± 19	78 ± 28
Valine	164 ± 15	157 ± 12

those in erythrocytes (Allen, 1960; McMenamy et al., 1960; Yunis
and Arimura, 1965).

Effects of Exercise on Amino Acid Concentrations

Plasma amino acids are extracted by the liver and utilized for energy metabolism via the gluconeogenic pathway (Mallette et al., 1969; Sladek and Snarr, 1971; Snell, 1974). While there is little reason to expect that the amino acids of the erythrocyte are unavailable for gluconeogenic energy production, there is little evidence for an active role.

Maintenance of glucose homeostasis and energy output during prolonged exercise requires an increase in gluconeogenesis since depletion of hepatic glycogen stores occurs during the initial 30-40 minutes of exercise. Ahlborg et al., (1974) demonstrated significant increases in splanchnic uptake of glycine, alanine, threonine, serine and proline following a 240 minute exercise period. The relative contribution of plasma amino acids to total glucose production rises from 5% at rest to approximately 12% after prolonged exercise. This data also suggests that alanine accounts for 55%, and glycine 21%, of this splanchnic amino acid exchange.

During exercise, the liver also releases amino acids (Felig and Wahren, 1971). Only the branch chain amino acids valine, leucine and isoleucine are released in significant amounts. The release of these amino acids from the liver suggests that they cannot be metabolized directly to glucose in the liver and that they can be more readily metabolized to glucose by other organs. The conversion of branch chain amino acids to alanine in muscle is consistent with this hypothesis (Odessy et al., 1974).

Glutamate and glutamine are extracted by the liver to about 25% of the extent of alanine (Felig et al., 1973; Garber et al., 1976a, 1976b; Karl et al., 1976). These amino acids are converted to alpha-ketoglutarate and subsequently to oxaloacetate for gluconeogenesis. The consistent uptake of glutamate and glutamine by the gastrointestinal tract suggests that the splanchnic uptake of these amino acids may be merely a direct reflection of extraction of glutamate and glutamine by the intestinal tract. This is a significant difference from alanine and glycine which are absorbed directly by the liver and actually released by the gastrointestinal tract.

UTILIZATION OF AMINO ACIDS BY THE LIVER AND KIDNEY FOR GLUCONEO-GENESIS

The Glucose-Alanine Cycle and Energy Production

It has become clear over the past ten years that gluconeogenesis has a significant role in energy metabolism (Daniel et al., 1977; Felig, 1973; Felig and Wahren, 1974; Felig et al., 1970).

The liver and to a lesser extent, the kidneys are the principle
sites of gluconeogenesis. Three amino acids account for more than
80% of splanchnic gluconeogenesis. Alanine has been identified
as the primary amino acid extracted by the splanchnic bed in both
the postabsorptive state and after prolonged fasting or exercise.
Alanine accounts for more than 50% of gluconeogenesis. Glycine
and glutamate/glutamine account for approximately 20% and 15% of
gluconeogenesis respectively. These amino acids are particularly
important as gluconeogenic substances during prolonged exercise.
This gluconeogenic pathway is depicted in Figure 1.

The branch chain amino acids are converted to alanine in the
muscle (Felig and Wahren, 1971; Paul and Adibi, 1978). Net synthe-
sis of alanine from amino acids occurs both by transamination and
by oxidative deamination. The resultant alpha ketoacid is con-
verted to malate and then to pyruvate. Transamination with glu-
tamate results in net formation of alanine. These gluconeogenic
entry reactions are summarized in Figure 2.

From these reactions, it is apparent that alanine and glutamine
are related through the common substrate glutamate. Thus, in
muscle metabolism, conditions resulting in increased pyruvate
availability yield increased alanine synthesis and decreased levels
of glutamine. Alternatively, factors increasing ammonia production
divert carbon and nitrogen from alanine formation toward glutamine
synthesis. Since glutamine is the principle substrate for renal
gluconeogenesis and alanine is the major substrate for hepatic
gluconeogenesis, muscle amino acid metabolism may have significant
regulatory impact upon gluconeogenesis in these organs (Felig
et al., 1977) (Figure 3).

Since the pyruvate required for net alanine synthesis does
not result from glucose metabolism in skeletal muscle, the decar-
boxylation of malate by malic enzyme represents a major source of
pyruvate formation (Goldstein and Newsholme, 1976). Thus, factors
affecting the flux of intermediates in and out of the tricarboxylic
acid cycle probably are significant in the regulation of alanine
and glutamine synthesis in muscle.

Studies of plasma amino acids during starvation suggest that
obese and normal subjects respond to fasting initially with a de-
crease in alanine and an increase in branched chain amino acids
(Felig et al., 1969, 1972; Haymond et al., 1974; Wahren et al.,
1973). The marked decrease in alanine is a result of hepatic de-
pletion of this substance for gluconeogenesis. The increase in
branched chain amino acids is due to hepatic release in an attempt
to provide the muscle with amino acid substrates for alanine and
glutamine synthesis so that renal and hepatic gluconeogenesis can
be maintained. Hypoalaninemia persists in prolonged starvation.
The regulatory importance of this hypoalaninemia is suggested by
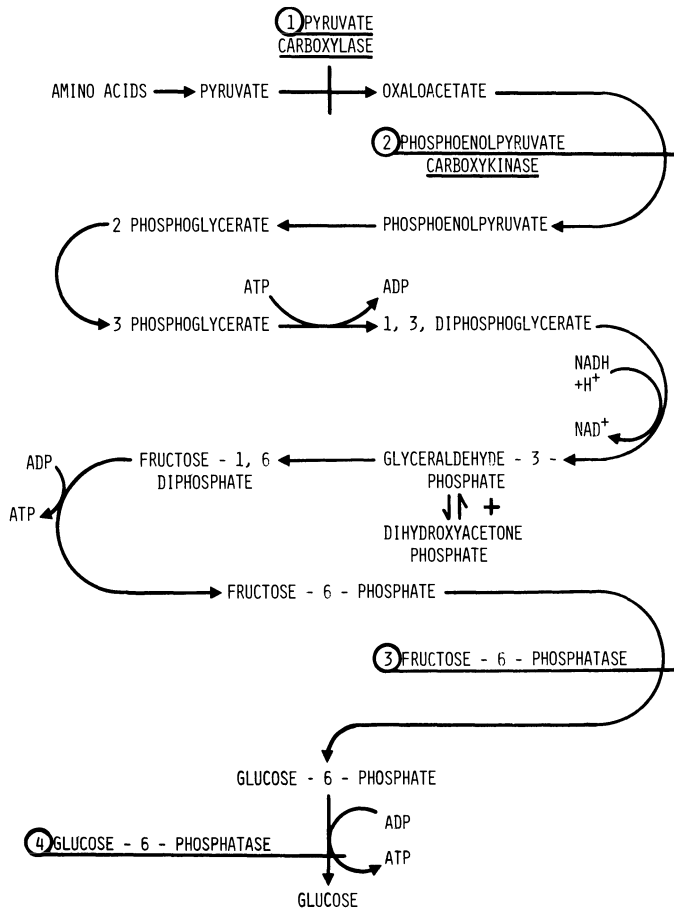
Fig. 1. The Gluconeogenic Pathway for Production of Glucose
From Amino Acids. Gluconeogenesis requires 4 enzymes
not present in the glycolytic pathway because of
thermodynamic irreversibility. They are numbered
1-4. All other enzymatic reactions are reversals
of glycolytic enzyme reactions due to substrate
availability.
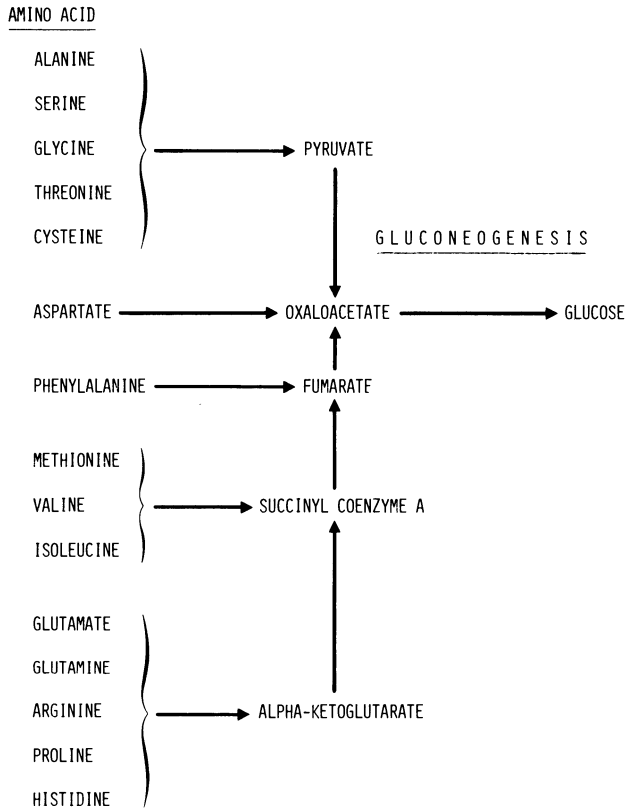
Fig. 2.   Entrance of Amino Acids Into the Gluconeogenic
           Pathway. Amino acids are converted to intermediates
           of the tricarboxylic acid cycle and thence to
           oxaloacetate. Oxaloacetate is converted to glucose
           via the gluconeogenic pathway of Figure 1.
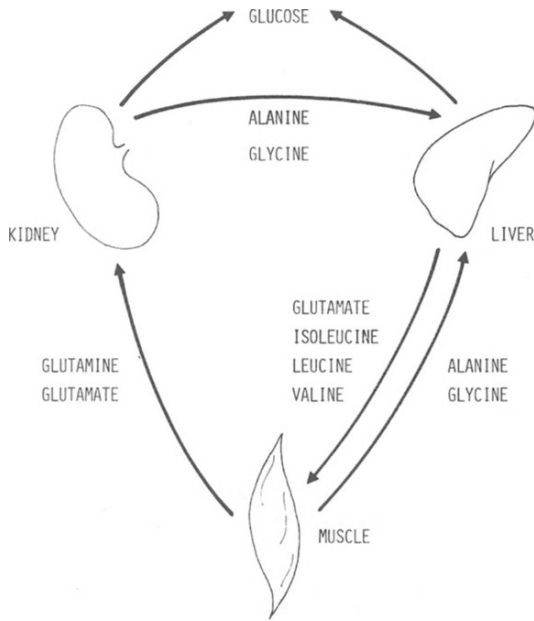
Fig. 3. Interorgan Transport of Amino Acids for Gluconeo-
genesis. Glucose is produced by the kidney and
liver during interorgan exchange of amino acids.
Branched chain amino acids are transported to the
muscle for conversion to alanine before returning
to the liver for gluconeogenesis. Glutamate is
converted by muscle to glutamine and transported
by the blood to the kidney for gluconeogenesis.

the fact that intravenous infusion of alanine into starved subjects
results in a prompt hyperglycemic response.

Energy Production From Amino Acids Other Than Alanine

In contrast to the behavior of other amino acids, the plasma
concentration of glycine, threonine and to a lesser extent, serine,
exhibit a delayed increase in starvation (Felig et al., 1969).
This is consistent with the hypothesis that as alanine is depleted,
secondary gluconeogenic amino acids become important metabolic
sources of energy. Although hepatic utilization of these gluconeo-
genic substrates is minimal, renal gluconeogenesis is augmented
by extraction of these amino acids (Mallette et al., 1969). The low
level of plasma serine may be due to its intermediate role in the
conversion of glycine and threonine to gluconeogenic substrates.

TRANSPORT OF AMINO ACIDS FOR GLUCONEOGENESIS

Active Transport of Amino Acids by the Blood

It has been generally accepted that plasma rather than erythro-
cytes is the vehicle of amino acid exchange between muscle and
gluconeogenic organs. This hypothesis stems primarily from in
vitro studies of equilibration times for amino acid transport
across the erythrocyte membrane (Felig et al., 1973). The results
of these studies have demonstrated uniformly slow equilibration
times. Initial suggestions of the possible importance of erythro-
cytes in amino acid transport stem from comparison of results
from studies using whole blood and studies using plasma. Van
Slyke and Meyer (1912) demonstrated a 2-3 fold increase in total
blood amino acid nitrogen following a meal, while others using only
plasma could not demonstrate such an increase. Further, the fact
that the cell/plasma ratio for amino acids is relatively constant
has been suggested as evidence of a diffusion controlled process
of amino acid accumulation by erythrocytes. However, if the pro-
cess were diffusion controlled, the cell/plasma ratio should be
approximately 1.0 for all amino acids. At least for arginine and
glutamine this is not the case. Arginine concentration in human
erythrocytes is quite low relative to plasma, less than 20%, while
glutamate concentrations in human erythrocytes are thirteen fold
higher than plasma concentrations (Levy and Barkin, 1971).

Aoki et al. (1972) demonstrated an apparent dynamic role for
the transport of glutamate. They infused insulin into the forearm
and examined the subsequent distribution of glutamate. Plasma
arterio-venous differences across the forearm muscles demonstrated
little glutamate uptake. However, whole blood arterio-venous
differences revealed that while resting muscle glutamate uptake
was small, insulin markedly increased the uptake of glutamate by
muscle from whole blood   This is consistent with the hypothesis

that in the resting state, much of the plasma glutamate is taken
up by the erythrocytes. However, during insulin infusion this
uptake by the erythrocytes is blocked and shunted into the muscle.
In addition to this redirection of glutamate, the erythrocyte
releases a portion of its glutamate to the muscle (Figure 4).
This uptake and release of glutamate is rapid (a single passage
through the muscle).

     The evidence for generalized amino acid transport by erythro-
cytes stems from research in dogs (Elwyn et al., 1972). All evi-
dence suggests a rapid exchange of amino acids between plasma and
liver. For glycine this has been calculated to be 350 μmoles/
min/kg of liver. Henriques et al., (1955) concluded that no mem-
brane barrier exists to diffusion of glycine between plasma and
liver. Thus, the principle determinant of this type of exchange
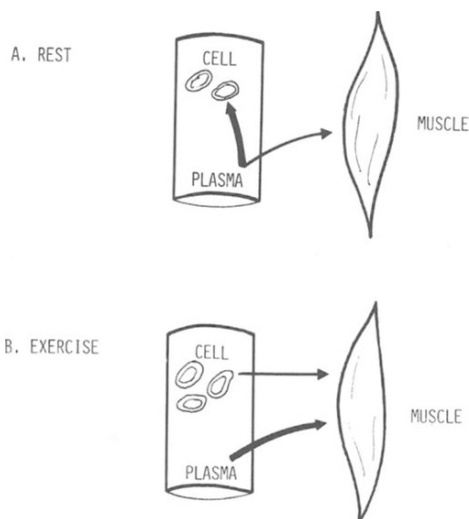
Fig. 4. The Role of Plasma and Erythrocytes in Maintenance
         of Muscle Glutamate. Muscle glutamate concentrations
         are maintained during exercise by a shift of accumu-
         lated glutamate out of both the plasma and the
         erythrocytes. The width of the arrow indicates the
         relative contribution (Aoki et al., 1972).

is blood flow. For glutamate flux, the liver output is negative
into the plasma but positive into erythrocytes. While glycine and
serine exhibit smaller fluxes than glutamate, the output of these
amino acids from liver are in opposite directions for cells and
plasma as is the case for glutamate. The magnitude and rapidity
of these fluxes, 40-100 µmoles/min/kg liver, makes the explan-
ation of these movements on the basis of a tissue → plasma →
erythrocyte sequence unsatisfactory. In order to explain the ob-
served changes in concentration solely in terms of red cell-plasma
amino acid transfer, very rapid changes in transport velocities
and enormous rates of production of glycine and serine must be
postulated. At the present, no evidence for these production
rates and velocities has been reported.

In vitro studies of amino acid transport have elucidated
several amino acid specific transport systems which are energy and
sodium dependent (Christensen, 1962). Additionally, passive trans-
port in erythrocytes has been proposed for some amino acids.
Passive transport is probably most important quantitatively in the
movement of amino acids between the luminal content of the small
bowel and the plasma postprandially. Although passive transport
of amino acids may account for a significant percent of amino
acid absorption from the gut, it seems likely that active trans-
port is the dominant factor in erythrocyte amino acid transloca-
tion. The fact that erythrocyte amino acid transport is frequently
spared in genetic disorders suggests that renal, intestinal and
erythrocyte active transport systems are genetically dissimilar
(Levy and Barkin, 1971; Marliss et al., 1972; Wahren et al., 1973).
This relative genetic stability may be a reflection of the quanti-
tative importance of active transport of amino acids in each
tissue.

Active transport of amino acids requires sodium (Christensen,
1962). The membrane bound amino acid carrier forms a ternary
complex with the amino acid and sodium. This complex traverses
the membrane and releases both the amino acid and the sodium. This
transport requires energy. Available evidence suggests actual
complex formation rather than an interaction of the sodium pump,
ATPase and the amino acid carrier complex. Arginine produces a
sodium independent inhibition of glycine transport apparently
because of the guanidinium portion of arginine occupies the sodium
site while the remainder occupies the amino and carboxyl sites of
the amino acid binding site. The observed stereospecificity of the
amino acid transport system suggests that amino acid side chain
recognition may also be a part of the system. While glutathione
has been suggested as a participant in erythrocyte amino acid
transport, in vitro evidence for this is lacking (Hagenfeldt
et al., 1978). Patients with glutathione synthetase deficiency
have presented divergent patterns of amino acid accumulation.
Thus, at present, there is no convincing evidence for a significant

role of glutathione in the active transport of amino acids by
the erythrocyte.

Developmental Changes in Blood Cell Amino Acid Transport

Mature human red cells accumulate both alanine and glycine,
but are apparently unable to transport methionine, valine, iso-
leucine, or leucine. Reticulocytes transport all of these amino
acids (Antonioli and Christensen, 1969). These differences are
probably the result of continuing protein synthesis in the reti-
culocyte. The erythrocyte continues to accumulate glycine for
glutathione synthesis and glycine is probably transported for
splanchnic or renal gluconeogenesis while alanine is transported
principally for splanchnic gluconeogenesis (Figure 3). Two sepa-
rate systems have been postulated for glycine transport (Christen-
sen, 1962). A transport agent serving principally for the trans-
port of alanine is entirely separate from that handling cationic
amino acids. While there is some evidence for sodium independent
amino acid active transport in the erythrocyte, the evidence for
this is equivocal (Antonioli and Christensen, 1969; Christensen,
1962). Christensen (1962) has concluded that interaction of
neutral amino acids in erythrocytes is restricted to a single
sodium dependent system.

The greatest difficulty in understanding the role of the
erythrocyte in inter-organ amino acid transport is the observed
dichotomy between in vitro amino acid transport rates and the rate
of transport observed in vivo. The transport rates in vivo are
magnitudes larger than the rates in vitro. Both direct transfer
of amino acids between erythrocytes and tissue cells, and rapid
increases in erythrocyte transport velocities upon entry of the
cells into the capillary beds have been proposed to account for
this discrepancy. Any rapid in vitro transfer of amino acids has
been obscured by experimental design in studies of erythrocyte
accumulation of amino acids. Therefore, the evidence for these
postulates is circumstantial and principally results from a failure
to provide more reasonable explanations. These hypotheses will be
difficult if not impossible to test.

SUMMARY

Gluconeogenesis from amino acids occurs primarily in the liver
and kidney. Alanine, glycine and glutamate are the principal
gluconeogenic amino acids but all common amino acids can potentially
be utilized for gluconeogenesis. Amino acid transport to the liver
or kidney occurs from the gut where amino acids are absorbed fol-
lowing a meal or from the muscle where amino acids are produced as
a result of transamination, amination or protein degradation.
Transport of amino acids in the blood is mediated by both the
plasma and the cells. Quantitatively, approximately 75% of amino

acid transport appears to be mediated by the plasma. Red blood
cells are responsible for the remaining 25%. Specific membrane
bound carrier proteins are responsible for the accumulation of
amino acids by the red blood cells. These carrier proteins re-
quire sodium and energy for proper function. While many specific
amino acid carrier proteins are lost during reticulocyte matura-
tion, alanine, glycine and glutamate carrier proteins are retained
in the erythrocyte. Translocation of amino acids from the erythro-
cyte to the tissue is very rapid, 300 µmoles/min. This observed
velocity precludes a sequence of transfer from the erythrocyte to
the tissue via the plasma by known transfer mechanisms. Direct
transfer between the erythrocyte and tissue has been proposed to
account for the observed velocities. However, the mechanism by
which this transfer occurs remains to be elucidated.

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*Summary papers presented at the National Academy of Clinical
Biochemistry symposium "Nutritional Elements in Clinical
Biochemistry", July, 1979.