THE STABILITY OF VITAMIN A IN HEMOLYZED BOVINE BLOOD

by

VICHIEN SAKORAMONKOL

A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

June 1962

APPROVED:

Redacted for Privacy

_____
Professor of Biochemistry

In Charge of Major

Redacted for Privacy

_____
Chairman of Department of Chemistry

Redacted for Privacy

_____
Chairman of School Graduate Committee

Redacted for Privacy

_____
Dean of Graduate School

Date thesis is presented __August 2, 1961__

Typed by Verna Anglemier

ACKNOWLEDGEMENTS

The writer wishes to express his sincere appreciation
and gratitude to several people who have contributed to
the completion of this thesis. Special acknowledgement is
due the late Dr. Joseph S. Butts, Head of Department of
Agricultural Chemistry and major professor, for his guid-
ance and suggestions. Special thanks also go to Dr. Paul
H. Weswig, the present major professor for his inspiration,
invaluable assistance, guidance and friendly criticism
throughout the course of the investigation and in the
writing of this thesis.

To Dr. Jesse F. Bone, Dr. Ralph Bogart, and Weldon K.
Johnston, Jr., the writer expresses his sincere thanks for
the permission and kind withdrawal of the blood from the
calves, for without their co-operation there would have
been no study.

Appreciation is expressed to Dr. Joseph R. Haag,
Dr. Albert V. Logan and Russell O. Sinnhuber, Associate
Food Technologist, for their discussions and suggestions
regarding the problem of this study.

The writer is grateful to Dr. Ian J. Tinsley for his
permission and kind advice in operating the Recording
Spectrophotometer.

Sincere thanks are given to other members in the

Department of Agricultural Chemistry who have in some way taken a part in this study.

Lastly, the writer would like to express his sincere thanks to the Thai Government for the permission to study and the financial support. Indebtedness and sincere appreciation are also given to Dr. Chang Ratanarat, Director-General of the Department of Science, Ministry of Industry, Bangkok, Thailand, for supporting and encouraging the writer throughout the entire study. Sincere thanks and appreciation are expressed to Mrs. Supis Sakoramonkol, the writer's wife, for her persistent encouragement.

TABLE OF CONTENTS

TABLE OF CONTENTS
(continued)

LIST OF TABLES

THE STABILITY OF VITAMIN A IN HEMOLYZED BOVINE BLOOD

INTRODUCTION

Vitamin A was one of the earliest vitamins to be
discovered. Its existence as a factor to correct the
ability to see in dim light dates back several thousand
years. The metabolic function of this vitamin in the
animal body is still not well understood. Night blindness
or nyctalopia, which is regarded as a specific symptom for
vitamin A deficiency in animals, is an important function
of this vitamin. It is elucidated nowadays that the main
factor for dark adaptation, the visual purple, is a pro-
tein complex of vitamin A. This is not the only function
of vitamin A since some animals die from vitamin A defic-
iency but not necessarily from night blindness. Vitamin A
is thought to be responsible for the general well being of
all parts of the body. Many syndromes such as xerophthal-
mia, epithelial keratosis, bone deformation are the
examples of general pathological effects influenced by
vitamin A, which will bring about injurious actions upon
the animal health.

The importance of vitamin A is quite obvious from the
report of Ensminger, Galan and Slocum (42, p. 18) who
state that 26.2% of all cattle afflicted with a nutritional

disease in the United States suffered from a vitamin A
deficiency. This disorder ranks second only to that of
bloat which accounted for 40.5% of all nutritional defic-
iency disease and ailments reported.

It seems to be possible that the epithelial keratini-
zation and the visual syndrome in deficient animals may
not be observed for some time. The observed morphological
lesion may thus be the final and distant manifestation of
an original lesion. A great deal of effort and research
has been aimed to study biochemical functions of vitamin A
including its distribution, breakdown, and transformation
products in the intact animals, by the application of
radio-isotope vitamin A as a tracer.

Most of the physiological studies with vitamin A are
based on the biological method in which chicks, rats and
rabbits are usually used as test animals. Information de-
rived from these animals is expected to apply to farm ani-
mals and humans, but this may not be necessarily true.
Since large animals are not as available or convenient,
it is not surprising that information on them is still
quite incomplete.

The mechanism of Vitamin A in the blood system is
quite complicated and still remains to be worked out in
detail. Many species of animals have been injected with
vitamin A using different carriers in order to study the

mechanism but more information is still required. It has
been reported in current literature that large amounts of
carotene and vitamin A are rapidly destroyed by the body
within a short period of time after intravenous injection.
This work was further extended by Pollard and Bieri (110,
p. 359-366) who showed with rats that blood appears to be
the tissue primarily responsible for the destruction of
vitamin A. They found a high rate of destruction in the
blood of animals having a high reticulocyte count.

The main purpose of this paper is to study the
stability of vitamin A in the hemolyzed bovine blood and
to determine whether it will show the same phenomenon as
in the rat. The possibility of bovine blood containing
a vitamin A destructive system is of obvious importance.

LITERATURE REVIEW

The Relationship of Carotene and Vitamin A

Carotene is pro-vitamin A

The general interpretation of the term "pro-vitamin A" is a compound which does not itself act as a vitamin but assumes the character of a vitamin after minor changes in its chemical structure. The organism itself is capable of carrying out the chemical changes necessary for the transformation into the vitamin. The evidence that carotene functions as a pro-vitamin A was discovered early in the twentieth century. Osborne and Mendel (106, p. 187-200) showed that numerous green vegetables were highly active as a source of vitamin A. Steenbock and Boutwell (123, p. 81-96) observed that yellow maize contained enough of the fat-soluble vitamin to allow growth in young rats. Steenbock et al. (124, p. xxxii) claimed that the feeding of re-crystallized carotene induced growth in rats on a diet devoid of the fat-soluble vitamin.

Moore (96, p. 380) suggested that carotene may be the precursor of vitamin A and may give rise to vitamin A in vivo. This suggestion was based on the evidence from the antimony trichloride reaction. However, Drummond, Channon and Coward (35, p. 1065) reported that a specimen of

carotene, re-crystallized four times, did not show the
growth-promoting activity of vitamin A. This evidence
was also supported by the work of Duliere, Morton and
Drummond (36, p. 321 T). The controversy was reconciled
by Hume and Smedley-Maclean (66, p. 290-292) who found
that the dissolved carotene in natural oil is stable
whereas it is destroyed rather rapidly in oleic acid or
ethyl oleate which was used by Drummond and co-workers.
Therefore the carotene was presumably destroyed before it
effected a cure in rats.

In order to obtain proof of the formation of vitamin
A from carotene Moore (97, p. 696-702) fed rats a diet
deficient in vitamin A until their liver extract gave no
blue color with the antimony trichloride reagent. After
feeding large doses of carotene the presence of stored
vitamin A in the liver indicated that dietary carotene was
converted into vitamin A. Similar results were reported
for chickens by Capper, McKibbin and Prentice (26, p. 265-
274).

Site of conversion of carotene to vitamin A

It was proposed by Moore in 1930 (97, p. 696-702)
from his experimental evidence that the liver be considered
as the main site of the metabolic conversion. Olcott and

McCann (105, p. 185-193) found spectrophotometric evidence
for the formation of vitamin A when fresh liver tissue or
an aqueous extract of liver tissue from vitamin A deficient
rats was incubated with a colloidal solution of carotene.
They proposed the presence of an enzyme carotenase which
was responsible for converting carotene into vitamin A.
Repetition of this work by numerous investigators resulted
in both confirmation and denial with the opposing view-
points about evenly divided. Attempts to extract the
proposed enzyme carotenase were also unsuccessful.

Mattson, Mehl and Deuel (87, p. 65-73) obtained the
evidence that vitamin A appeared in the intestine wall
before it was found in the liver when carotene in oil was
fed to vitamin A deficient rats. They concluded the in-
testinal wall was the site of conversion of carotene to
vitamin A in the rat. The conversion of vitamin A aldehyde
to vitamin A in the intestinal wall reported by Ball et al.
(11, p. xxiv) supported the possibility that the conversion
of carotene might take place in the same site. Alexander
and Goodwin (1, p. 421-423) demonstrated that the oral
administration of carotene to conscious rats with
cannulated intestinal lymphatic vessels showed a marked
increase in the vitamin A content of the lymph. No caro-
tene was observed in the lymph. They indicated that the

intestinal wall is the site of the conversion. Elliot
(40, p. 711-712) observed increased amounts of vitamin A
in the blood plasma taken from various sites along the
intestinal tract of dairy calves following the ingestion
of a carotene concentrate.

In order to elucidate the importance of the various
possible sites for the conversion of carotene, Bieri and
Pollard (18, p. 32-44) demonstrated the increasing of
blood vitamin A after the intravenous injection of caro-
tene into rats from which the small intestine had been
removed. This increasing also occurred in nephrectomized
or partial hepatectomized rats. They concluded that caro-
tene can be converted to the vitamin A elsewhere than in
the intestine. Kon, McGillivray and Thompson (70, p. 244-
267) with similar experiments in rats, rabbits, and calves
supported this view. Carotene when injected into calves
is converted only to a very limited extent into vitamin A.
Many investigators reported that the appearance of vitamin
A in the rat after injection of carotene is in no way
affected by removal of the liver (89, p. 126-134), stomach,
small intestine, large intestine, pancreas, kidneys,
adrenals, or gonads (143, p. 169-175) or by complete
removal of all lung tissue (144, p. 44-47). Therefore it
would appear from all evidence available that there is no

organ or tissue particularly involved in the conversion.
It might be possible that the small intestine is the
common site for the conversion and the liver and probably
other tissues may be alternate sites.

All of these experiments have been done in vivo.
Many attempts have been made to demonstrate the conversion
in vitro but the results were conflicting. The difficul-
ties were due to the uncertainty of detecting vitamin A
in the small amounts claimed to be produced. Wiese, Mehl,
and Deuel (141, p. 75-79) found after incubating the
washed intestine of rats with an aqueous carotene and
tocopherol dispersion, the presence of vitamin A by the
Carr-Price reagent. Rosenberg and Sobel (116, p. 320-325)
confirmed the above experiment and observed increasing
vitamin A in the intestine by using the destructive ir-
radiation technique for vitamin A determination. Similar
results were also obtained in calves by Stallcup and
Herman (122, p. 237-242) and in sheep by McGillivray (88,
p. 370-376).

In contrast Bieri and Pollard (17, p. 402-411) were
unable to find any increase in the vitamin A in the rat,
rabbit or calf by similar experiments. Negative results
were also reported in the rat by Kon and Thompson (69, p.
114-119) using the perfusion technique. In 1959 Worker

(145, p. 400-418) was unable to demonstrate in vitro the conversion of carotene into vitamin A either in the perfused intact animal or in tissue slices or in homogenates of body wall, intestines, kidney, liver, or lung of the rat or guinea pig. Thus up to now there is no undisputable evidence to show in vitro a site of conversion of carotene to vitamin A.

Mechanism of the conversion

After consideration of the formula of beta-carotene and vitamin A many authors regarded the conversion reaction as a hydrolytic fission at the central double bond of beta-carotene. Actually this reaction is found to be unsuccessful and leads to the suggestion that oxidation takes place at the central bond resulting in the formation of vitamin aldehyde which is further reduced to vitamin A alcohol. Chemically such a reaction occurs when beta-carotene is oxidized with hydrogen peroxide in the presence of a suitable catalyst.

Glover and Redfearn (50, p. xv-xvi) showed that carotene was oxidized stepwise from the end of the molecule by a type of beta-oxidation to form vitamin A aldehyde. The methyl branches of the fatty acid are oxidized in vivo when the methyl group is in the alpha position but not in

the beta position to the carboxyl group (78, p. 235-246).
Since vitamin A aldehyde contains the methyl group in the
beta position the latter is not oxidized but is easily
reduced by alcohol dehydrogenase. Fazakerley and Glover
(45, p. 38P-39P) after further study of this reaction
suggested that the beta-12' apo carotenoid compound is not
degraded by the beta oxidation to vitamin A because the
beta-14' apo carotenoid compound is not the intermediate
product of vitamin A. The beta-12' apo compound might be
metabolized by another enzyme system which removes the
five terminal carbons as a single unit.

Fishwick and Glover (46, p. 36P-37P) demonstrated
that C^{14}-labeled-carotene which was given orally to vitamin
A deficient rats approximately 50% to 70% of the dose was
unabsorbed. Of the absorbed portion the C^{14} beta-carotene
was rapidly degraded during its absorption across the
intestinal wall. They indicated that the C^{14} beta-
carotene was attacked at some position rather than the
central double bond and the formation of vitamin A at
least involved in part some other system than beta-
oxidation. Krause and Sanders (73, p. 549-551) confirmed
these findings and showed that carotene is not entirely
converted into vitamin A. They suggest that carotene
might be directed into other metabolic pathways.

The Absorption of Vitamin A

Mode of absorption

It was suggested by Eden and Seller (38, p. 264-267)
that the major part of the absorption of vitamin A in
ruminants and rats takes place in the upper part of the
intestine. An increased vitamin A level was found both
in the systemic and portal vein blood. Larger increases
were observed in the intestinal lymph. Thompson, Ganguly
and Kon (131, p. 50-78) found that the concentration of
vitamin A is highest in the middle portion of the small
intestine and that the lymphatic system is the route of
transportation of vitamin A. Ronning and Knodt (115, p.
283-291) working with calves reported the most active
absorption of vitamin A appears in the upper two-third
of the small intestine.

Gray, Morgareidge and Cawley (53, p. 67-74) found
that a vitamin A ester which was administered to the rat
was partially hydrolyzed in the intestine by an enzyme.
During the absorption the vitamin existed in the lumen of
the intestinal wall chiefly in the alcohol form. Similar
results were reported by Eden and Sellers (39, p. 261-266)
using calves and sheep. The existing vitamin A alcohol
is further absorbed by the intestinal epithelium and is

resynthesized to the ester form from which it passes into the intestinal lymph as the esterified vitamin A. It was reported by Mahadevan, Krishnamurthy and Ganguly (86, p. 371-375) that the vitamin A esters of the lymph as well as the blood and the liver of the rat are formed by long chain fatty acids. The ester which is present in the normal rat liver probably is a palmitate ester (52, p. 301-307).

Several other tissues as well as the intestinal wall have been reported capable of esterifying vitamin A. The intracellular spaces of the subcutaneous tissue also contain an enzyme capable of esterifying vitamin A alcohol (49, p. 109-114). Both Krause and Powell (72, p. 57-62) and High and his co-workers (62, p. 556; 63, p. 338) demonstrated that the homogenate of the intestine and kidneys are able to esterify vitamin A whereas blood, liver homogenates and their cellular components show very little esterification. The nephrectomized rat exhibited a slow rate of esterification. However, esterification still proceeded in all experimental groups of animals.

Krinsky (74, p. 881-894) obtained a cell-free enzyme from various eye tissues which was capable of esterifying vitamin A alcohol. The enzyme is concentrated in the pigment epithelium where it is found in the particulate

fraction. A product of the reaction is a long chain fatty
acid ester of vitamin A which is capable of being hydro-
lyzed by the retina. Pollard and Bieri (111, p. 9-12)
reported that a pancreatic enzyme from the rat and chick
utilizes free fatty acids in the esterification of vitamin
A. Murthy et al. (103, p. 482) recently studied the fatty
acid specificity for the esterification of vitamin A by
intestinal and pancreatic enzymes in the rat. Neither
tissue showed any preference for unsaturation and it was
effectively esterified with fatty acids containing more
than 12 carbon atoms by both tissues.

The hydrolysis of stored vitamin A ester in the liver
has been investigated both in vivo and in vitro. Krause
and Alberghini (71, p. 396-400) in order to explain the
predominance of the alcohol form in the blood, found a
hydrolytic factor existing in the serum and plasma of
blood. McGugan and Laughland (90, p. 428-434) using
vitamin A acetate and liver homogenates demonstrated the
vitamin A esterase activity in rat liver tissue. High
and his co-workers (62, p. 556; 63, p. 388), Krause and
Powell (72, p. 57-62) and Laughland (79, p. 95-99) held
that when the vitamin A acetate, caprylate, laurate and
palmitate were hydrolyzed by liver homogenate the palmitate
ester exhibited the least followed by the laurate with the

acetate having the greatest rate of hydrolysis. The rate
of hydrolysis of vitamin A acetate was greatest in the
liver, intermediate in the blood and least in the kidney.
This suggested that during the metabolism of vitamin A
the natural ester is converted to shorter chain esters
before the final hydrolysis. The liver is the chief site
of this process. The liver from vitamin A deficient rats
hydrolyzes these esters as readily as those from rats
which have received supplementary vitamin A.

Krause and Powell (72, p. 57-62) reported that the
liver mitochondria and nuclei are the most active in their
ability to hydrolyze vitamin A acetate. This is contra-
dictory to the reports of Ganguly and Deuel (47, p. 120)
that the activity is quantitatively present in the micro-
somal fraction and that the nuclei, mitochondrial, and
supernatant fractions are inactive. Krishnamurthy,
Seshadri Sastry and Ganguly (77, p. 391-394) studied the
properties of vitamin A esterase in homogenates of rat
pancreas. They demonstrated that neither blood nor liver
hydrolyzed the ester whereas the pancreas did it readily.
Blood vitamin A ester is probably derived from the liver
and is hydrolyzed by tissues like the pancreas, small
intestine, spleen, and kidneys but not by the liver or
blood. This maintains the blood and liver vitamin A

alcohol levels.

Vitamin A and protein relationship

The mechanism of storage and the mode of release of
the vitamin are still controversial subjects. As pre-
viously mentioned, liver homogenates are unable to hydro-
lyze the natural or palmitate esters of vitamin A. It
is possible that the hydrolysis of these esters does not
take place in the liver. Probably vitamin A ester leaves
the liver as such and is converted to the alcohol form in
the circulating blood system. A mechanism of the vitamin
A and carotenoid transport and its association with serum
protein is still not fully understood. Crook and
El-Marsafy (29, p. viii) reported that carotene was pri-
marily associated with beta-globulin. This is in contrast
to an earlier report by Dsialossynski, Mystowski and
Stewart (37, p. 63-69) that vitamin A and carotene were
associated with the albumin from the ammonium sulfate
fraction. Krinsky, Cornwell and Oncley (75, p. 113; 76,
p. 233-246) using several fractionation procedures includ-
ing precipitation with dextran sulfate and ultra centrifu-
gal floatation found that approximately 90% of the total
human plasma carotenoids are distributed between the alpha
and beta lipo-protein fractions. The vitamin A alcohol
was bound by a plasma protein but not by alpha lipo-protein

or albumin. Available evidence would suggest that the vitamin A is made soluble in the blood by forming complex substances with protein.

Erwin, Varnell, and Page (43, p. 373-375) by electrophoresis fractionation of bovine serum concluded that vitamin A and carotene were principally associated with albumin. However Garbers, Gillman and Peisach (48, p. 124-132) with the aid of an electrophoresis technique and labeled vitamin A reported that vitamin A alcohol is transported in rat serum and associated with the $alpha_1$ globulin and not associated with the lipo-protein or with the $alpha_1$ glycoprotein. They found in the serum a labeled component derived from $2\text{-}C^{14}$ vitamin A and associated with the $alpha_2$ globulin which is not extractable from the denatured serum. It is most likely a normal constituent of serum occurring in a concentration equivalent to 20-30 I.U. of vitamin A per 100 ml. of serum. This component is observed after the oral administration of labeled vitamin A to depleted rats. The amount of vitamin A in rat serum as determined by the Carr-Price reaction cannot always account for all the radioactivity present in the serum.

Efficiency of vitamin A absorption

The efficiency of the absorption of vitamin A may be
determined by many procedures. The measurement of the
vitamin A level in the liver or in the blood plasma after
the administration of large doses of vitamin A or its
precursor seems to be one of the methods most generally
used. The cerebrospinal fluid pressure method was also
applied to measure the status of vitamin A level in animals
by Moore and his colleagues (94, p. 684-689; 93, p. 649-
658; 95, p. 533-538). They found that the spinal fluid
pressure increased in calves suffering from avitaminosis
A. The vitamin A deficiency and low plasma carotene are
correlated with an increased cerebrospinal fluid pressure.
This measurement provided a fairly critical index of the
inadequacy of carotene intake and was applied to the study
of the carotene requirements of calves. During the winter
Guernsey calves required an intake of 34 ug. of carotene
per pound body weight to maintain a normal spinal fluid
pressure. Holstein and Ayrshire calves required 30 ug.
of carotene per pound body weight.

It is evident that more reliable information may be
obtained from the liver as compared with blood plasma.
In animal experimentation it is necessary that the vitamin
A concentration of the liver be depleted or present only

in a very small quantity. After dosing with a source of
the vitamin, the amount of vitamin A in the liver may be
determined in order to calculate the efficiency of vitamin
A absorption. In dairy cattle vitamin A in the liver may
be determined by liver biopsy (21, p. 747-752). A blood
plasma measurement is useful in investigations with large
animals or human subjects.

It has been shown by Lemley et al. (82, p. 53-64)
studying the absorption and storage of vitamin A in the
liver of the rat that the efficiency is affected by the
magnitude of the dose. After the maximum absorption was
reached, further increase in the amount of vitamin admin-
istered was accompanied by a decreased efficiency of ab-
sorption. The method of administration profoundly
influenced the efficiency. The oral method was found to
be most effective in producing liver storage. Subcutaneous
and intramuscular administration are only 35% and 2%
respectively, as effective as the oral route. Wide varia-
tions of the storage of vitamin A in the liver were found
depending both on the form of vitamin A fed and the char-
acter and quantity of the diluent (140, p. 233-250). The
storage of vitamin A in the liver of rats also increased
when tocopherols were given with vitamin A for an extended
period (83, p. 205-218).

The increase in vitamin A in blood plasma at a defi-
nite interval after dosing represents only a small frac-
tion of the total amount which has been ingested. The
only possibility in applying the blood plasma method is
based on the assumption that the total absorption is pro-
portional to the increase observed in the plasma vitamin
A. It remains possible that the slow removal of the vita-
min from the blood plasma by the liver may give an indica-
tion of the rapid absorption from the intestine.

Davis and Madsen (32, p. 135-146) reported that vita-
min A deficiency in cattle may be detected by blood caro-
tene and vitamin A analysis. The carotene and vitamin A
content of blood plasma are dependent on the carotene in-
take and the previous storage. However, the blood plasma
measurement is also influenced by many factors similar to
the liver storage procedure. Thomas and his co-workers
(130, p. 679-686) indicated that higher levels of plasma
vitamin A sometimes occur when calves are fed on a skim
milk, vitamin A deficient diet than when they are fed a
diet adequate in vitamin A. It is possible that the body
stores were called upon to supply the necessary vitamin A
when the dietary source was omitted. However, it may
indicate that there are some factors present in skim milk
altering the release mechanism. Thus on certain dietaries

the plasma vitamin A level is not a reliable indicator of the intake.

Several attempts have been made to study the relationship between blood and liver levels of vitamin A and carotene in various animal species. In a recent paper Diven et al. (33, p. 1632-1637) briefly summarized the foregoing papers and described the interrelationships of vitamin A and carotenoids in bovine plasma and liver. By means of statistical methods using the arithmetic and log functions a positive relationship occurred in pairs of plasma vitamin A and plasma carotenoids, hepatic vitamin A and hepatic carotenoids, plasma carotenoids and hepatic carotenoids, and hepatic vitamin A and plasma carotenoids. Little or no relationship was found in pairs of plasma vitamin A and hepatic vitamin A, in plasma vitamin A and hepatic carotenoids.

The Stability of Vitamin A

Effect of light and air

Vitamin A as well as its precursor is chemically rather sensitive to oxygen and light. It was shown by Evers (44, p. 556-565) that cod liver oil loses its vitamin A activity on exposure to light or oxidation. Sunlight particularly is more destructive than ultraviolet light.

Hydroquinone does not prevent the destructive effect of light. Smith et al. (118, p. 207-212) reported that the exposure of vitamin A containing concentrate or oils to ultraviolet irradiations of the wavelengths over 300 mu, the optical density at 328 mu progressively decreased. However, if the irradiated solutions were kept in the dark the optical density tended to increase towards the original value. The greater the original decomposition the less complete the recovery. They suggested that the vitamin underwent a reversible photochemical isomerization.

Bolomey (19, p. 323-329; 20, p. 331-335) did not notice any restoration of the optical density in this treatment. The time necessary to decompose 50% of vitamin A under aeration at 100°C in shark liver oil or vitamin A preparations varied from 52 to 175 minutes. The length of the induction period varied inversely with the temperature of the aeration. The irradiation of vitamin A containing oils produce a secondary photo-oxidation that is responsible for the changes in the absorption spectrum of vitamin A.

Influence of trace elements

The influence of trace elements on the stability of vitamin A has been studied by many investigators concerned primarily with stored poultry feeds as they always contain

free trace minerals in their mixed diet. Miller, Joukovsky
and Hokenstad (92, p. 200-202) reported that the addition
of manganese sulfate to mixed feeds destroyed the vitamin
A and vitamin D values. After 56 days of storage, all of
the vitamin A was destroyed when the manganese sulfate
content of the mixed feed was 0.5% of the mixture.

Halverson and Hart (57, p. 415-427) found that adding
moderately high levels of the common trace minerals man-
ganese, iron, copper, and cobalt salts to a white corn
feed supplemented with cod liver oil, caused an extremely
rapid loss in the vitamin A activity. This loss could be
prevented by adding the minerals in dried gelatin mixture
rather than in the free form. Continuing this experiment
Halverson and Hendrick (58, p. 355-359) observed that the
addition of manganese sulfate alone to the poultry rations
did not materially effect the loss on the stored samples
when compared with samples without the trace elements.
This is contrary to the findings of Miller, Joukovsky and
Hokenstad as previously indicated. However, the addition
of all of the trace elements at a higher level as in a
feed concentrate caused an increase in the loss with all
rations especially after 150 days storage.

Bäck (8, p. 334-335; 9, p. 60-74) found that the
stability of vitamin A in fish liver oils is adversely

affected by traces of metals. The influence of the different concentrations of some trace elements cause the acceleration of destruction. The metals investigated were aluminum, copper, iron, nickel, tin and zinc. The ascending order of the vitamin A destruction was zinc, tin, aluminum, nickel, copper and iron. The most rapid destruction of vitamin A was caused by copper at 1 p.p.m. and 10 p.p.m. and by iron at 100 p.p.m. When the metallic ion was added as a stearate the destructive effect was less than when it was added as a chloride. Citric acid was found to act as a good stabilizer of vitamin A.

The effect of sodium bentonite which is usually added to the feed at the level of about 2% to improve pellet characteristics and to exert a lubricating action on the die of the pelleting machine was investigated by Laughland and Phillips (80, p. 593-599; 81, p. 610-620). Its addition to the diet severely limited the ability of the rat to store vitamin A in the liver. Sodium bentonite and vitamin A form a blue adsorption complex. The chemical alteration which occurs in the vitamin A molecule is the formation anhydrovitamin A. When gelatin is added to a commercial mineral salt mixture there is a great decrease in the vitamin A destruction as influenced by trace elements (31, p. 107-112). The stability of vitamin A was greater when a waxed sealed vitamin A supplement was used

as compared with oil supplementation both with or without
added trace minerals (58, p. 355-359; 113, p. 603-608).

Influence of other factors

Various factors involving stability of vitamin A in
stored feed have been extensively investigated. Vitamin A
added as cod liver oil to mixed rations is not stable in
storage in the usual sealed container at different mois-
ture levels. Vitamin losses in both sealed and unsealed
samples increased with both temperature and length of
storage (57, p. 415-427; 113, p. 603-608). Increasing
the concentration of the vitamin results in a marked in-
crease in its stability (139, p. 1146-1150). The stability
of the vitamin is greater in a chicken mash of a medium
particulate size than in one of coarse particles (31, p.
107-112). Vitamin A in cod liver oil is less stable in
various cereal products than in fish meal or liver meal.
Vitamin A palmitate is superior to cod liver oil when
added to a mixed meal.

Effect of vitamin E

It is recognized that fats and oils from plant prod-
ucts are protected from oxidation by tocopherol. Hence,
animal fat may be expected to be protected by biological

antioxidants in vivo during the period of storage and
digestion. The effect of vitamin E on the stability of
vitamin A was shown by Moore (98, p. 1321-1328) who re-
ported that the vitamin A reserves of the rat on a diet
deficient in vitamin E were much lower than those receiv-
ing supplements of vitamin E. The growth promoting abil-
ity of vitamin A was enhanced by the addition of vitamin
E (60, p. 303-311). Buxton (24, p. 225-232) studying the
tocopherols as antioxidants of vitamin A in fish liver
oil reported that beta and gamma tocopherols were found
to be effective anti-oxidants for retarding peroxidation
and at 0.10% markedly increased the stability of vitamin A.

The antioxidant behavior of vitamin E with reference
to vitamin A has been studied by many animal laboratories.
When vitamin E was fed to rats along with vitamin A the
storage of vitamin A in the liver and kidney was unaffected.
On the contrary, when carotene was fed instead of vitamin
A a decrease in liver storage of vitamin A resulted (125,
p. 120-126). Tappel (127, p. 223-225; 128, p. 473-485)
demonstrated that alpha-tocopherol inhibited the oxidation
of vitamin A and carotene in hematin-catalyzed oleic acid
oxidation using the manometric and spectrophotometric
assay. The other phenolic antioxidants, nordihydro-
guaiaretic acid, propyl gallate and butyrated hydroxy-
anisole were also effective inhibitors of linoleate

oxidation catalyzed by hemoglobin and cytochrome c. He favored the concept that tocopherol functions in vivo as an inhibitor of the auto-oxidation of unsaturated fatty acids.

High (64, p. 681-686; 61, p. 456-462) found that when the vitamin A deficient rats were fed relatively large amounts of alpha-tocopherol or other antioxidants plus moderate amounts of carotene in cottonseed oil the vitamin A deposition was decreased about 50% to 60% as compared with the control. Thus the antioxidants had no effect on the utilization of vitamin A. He concluded that the mode of action of vitamin E and other antioxidants is concerned with their antioxidant property. The presence of large amounts of these substances may suppress the oxidation processes which are probably involved in the enzymatic conversion of carotene to vitamin A.

Vitamin A Oxidative Products and Their Biological Functions

Vitamin A aldehyde (retinene)

Vitamin A aldehyde was first recognized by Wald (138, p. 351-371) during his extensive work with the frog retina. He found that the dark adapted retina contained a trace of vitamin A which could be extracted in the dark

with homopolar organic solvent like benzene without injur-
ing the visual purple. If the retina was exposed for a
short time to light, further extraction with organic sol-
vent now produced a greenish yellow pigment which exhibited
carotenoid properties different from any reported before
that time. He called that substance retinene which was
proved to be the same compound as vitamin A aldehyde by
Morton (99, p. 69-71).

This compound was prepared from vitamin A alcohol by
Oppenauer oxidation with aluminum isopropoxide in the
presence of acetaldehyde (67, p. 194), by direct oxidation
of vitamin A dissolved in light petroleum ether with
potassium permanganate in dilute sulfuric acid (100, p.
405-406), or with manganese dioxide and standing in a dark
place for a few days (12, p. 516-523). The vitamin A
aldehyde after purification by chromatography and re-
crystallization yielded a large orange needle-like crystal.
The absorption maxima in cyclohexane is at 373 mu and the
blue colored solution with antimony trichloride gives a
single absorption band at 664 mu. The reactions of
retinene with amino compounds and proteins yields yellow
or red compounds which resemble the first breakdown
products of visual purple (10, p. 304-307).

The biological importance of retinene in vision was
further elucidated by Wald and his colleagues on

demonstrating the enzyme system by which oxidations and
reductions between vitamin A and retinene are effected.
The role of the cis, trans, isomerism of retinene in
rhodopsin formation was also taken into consideration.
Hubbard and Wald (65, p. 269-315) proposed a cyclic iso-
merization of the rhodopsin system and concluded that the
retinene is liberated from the rhodopsin by the effect of
light in all-trans form and combined into rhodopsin in the
neo-retinene b form which is the isomeric form of retinene.

Vitamin A aldehyde in the body is easily reduced to
vitamin A alcohol. This was shown by Glover, Goodwin, and
Morton (49, p. 109-114) who found that after the oral
administration of vitamin A aldehyde to rats, some portion
remained unchanged in the stomach and in the small intes-
tine. No vitamin A aldehyde was found in the walls of the
intestine or in the liver but they did contain vitamin A
in the esterified form. After subcutaneous injection of
retinene, vitamin A was also found both in the tissues
near the site of the injection and in the liver.

Armes et al. (4, p. 174) reported that in humans,
rats, and chicks the conversion of retinene into vitamin
A alcohol or vitamin A ester is rapid and efficient.
About 30% of the ingested vitamin A aldehyde was destroyed
in the intestine; none was found in the blood of humans
or in the liver or feces of rats. Massive doses

of vitamin A aldehyde caused toxicity paralleling that of
the other forms of vitamin A. Armes, Swansen and Harris
(3, p. 4134-4136) showed that the biological activity of
all-trans vitamin A aldehyde estimated from the measure-
ment of rat growth and liver storage was found to have the
potency on a molar basis of about 91% of the activity of
the all-trans vitamin A acetate.

Vitamin A acid

Vitamin A acid has not yet been isolated from natural
sources although it could conceivably be formed from vita-
min A. Arens and Van Dorp (5, p. 190-191) synthesized
pale yellow crystals of vitamin A acid from the condensa-
tion of beta-ionone with gamma, bromo-crotonic methyl
ester in the presence of zinc. A beta-ionylidenecrotonic
acid was then converted to the corresponding C_{18} ketone
by the action of lithium methyl and hence to vitamin A by
treatment with bromo-acetic acid and zinc in benzene solu-
tion followed by dehydration with anhydrous oxalic acid.
If given orally dissolved in arachis oil to rats it has
about one-tenth the biological activity of vitamin A.
When injected as the sodium salt, the activity is about
one-half the potency of vitamin A. However, if the sodium
salt of crystalline vitamin A acid is given orally in an
aqueous solution the response is equal to that of vitamin

A itself given orally in oil (136, p. 60).

Since vitamin A acid is biologically active it is possible that this substance could undergo reduction in the body to vitamin A alcohol. To check this point Arens and Van Dorp (6, p. 622-623) gave massive doses of the sodium salt of vitamin A acid either administered orally or subcutaneously to vitamin A depleted rats. The vitamin A contents of the liver proved to be zero; hence, vitamin A acid itself exerted its own biological activity. Wodsak (142, p. 672-675) reported that vitamin A acid was not converted to vitamin A in the blood and disappeared rapidly after intravenous injection into rats. Vitamin A acid was reported to be more stable than vitamin A and had a greater stability in plant oils and fats than in beef fat.

A recent study of Dowling and Wald (34, p. 587-608) is of great theoretical interest concerning the biological function of vitamin A acid. They found that when weanling rats were supplied with vitamin A acid they grew as rapidly as those with vitamin A alcohol except for visual diffi-culties. Moreover, the acid immediately restored the growth of depleted animals. It was obvious that for growth and general health the acid was as effective as the alcohol. The liver stores of vitamin A of rats supplied with vitamin A acid declined as rapid as those on a

deficient ration. When the acid supplement was removed from the ration, the rats stopped growing and became severely deficient in a few days. No detectable amounts of either vitamin A alcohol or vitamin A acid were found in the liver.

It would appear that vitamin A acid is very rapidly destroyed and cannot be reduced to the aldehyde or the alcohol. Since retinene is needed to synthesize visual pigments, animals kept on vitamin A acid become highly night-blind though apparently in good condition. The failure to form visual pigments also has specific anatomical consequences. The outer segments of the visual cells deteriorate followed by the loss of almost all the cells in an otherwise normal retina.

They conclude that vitamin A is commonly stored in the tissue and principally in the liver as an ester. It is transported in the blood mainly as the free alcohol. Vitamin A is oxidized in the retina to retinene which is rapidly removed and combined with opsin to form rhodopsin. In the liver the alcohol is also oxidized to the acid which is either rapidly converted to an active product or is rapidly used in its metabolic function of growth and tissue maintenance. The acid may be the important form of vitamin A for these general functions.

Thompson and Pitt (132, p. 672-673) found that the vitamin A acid given orally as the sodium salt produced typical signs of hypervitaminosis in rats with a lower dosage and a shorter time than did vitamin A itself. They suggested that the acid form is more nearly like the systemically active form of the vitamin. Hypervitaminosis is produced by by-passing the normal control on the supply of active vitamin A to the tissues. Verandani et al. (137, p. 452) have been unable to detect or trap vitamin A acid as a metabolite of vitamin A. They suggested that both the alcohol and the acid may be the precursors of a common functional compound. With the intraperitoneal injection of radioactive vitamin A acid and vitamin A alcohol into deficient rats, the highest percentage of radioactivity was found in the liver from vitamin A and in the small intestine from vitamin A acid. The eye contained no radioactivity from vitamin A acid which confirmed the work of Dowling and Wald on the function of vitamin A acid in visual pigments.

Other oxidative products of vitamin A

It has been long known that vitamin A is unstable due to the influence of light and air. It was presumed that many oxidative products occurred with the formation of absorption bands moving towards shorter wavelengths of

vitamin A. On the contrary, the oxidation of vitamin A aldehyde which takes place at the end of the side chain produces an absorption band shifted towards the longer wavelengths.

Halpern (56, p. 621-625) reported that the saponifiable fraction of fish liver oil which was oxidized for a period of 248 hours in air at 75°C developed absorption bands at 280 mu. Bolomey (20, p. 331-335) noted the absorption maxima at 310-312, 294-296, 284-286, and 274-275 mu of vitamin A acetate in tri-acetin which was aerated or stored. Groot (54, p. 185-190; 55, p. 871-878) observed bands of 220, 273 and 310 mu when vitamin A acetate was dissolved in ethanol and was aerated for 10 minutes. On exposure to a few hours of diffused daylight the maxima appeared at 273, 280 and 310-312 mu. Spectroscopic changes occurred more rapidly when the solution was exposed to direct sunlight or to irradiation by a mercury arc lamp.

Troitskii (134, p. 485-489) compared the oxidative products formed by exposing vitamin A to air for a period of 20-40 days with the epoxide of vitamin A prepared by the action of perbenzoic acid in ether solution on vitamin A. He showed that the auto-oxidation of vitamin A gives substances similar to the epoxide by the absorption studies made with the antimony trichloride reaction. Two

chromogens with absorptions of 570 and 420 mu were also
obtained with auto-oxidation. The probable suggested
change occurred from the vitamin A epoxide to the chromo-
gen at 570 mu and then to the chromogen at 420 mu.

Destruction of vitamin A in blood

It was found by LePage and Pett (84, p. 747-761) that
88% to 96% of orally administered vitamin A cannot be ac-
counted for in the blood and feces of human subjects as
vitamin A. This large amount of vitamin A cannot be con-
sidered to be withdrawn and stored in the tissue. Since
it was shown by Baumann et al. (13, p. 705-715) that vita-
min A in rat tissue was found to be only 10-20% of the ad-
ministrative dose, they pursued the destructive products
of vitamin A in blood by spectrographic examination of the
purified extracts of blood after the administration of
vitamin A. The substance seemed to be an oxidative product
of vitamin A. Two possibilities should be considered,
either the oxidative product is produced in the blood
after absorption in which case some must be excreted again
or it is produced in the gastrointestinal tract and part
of it absorbed.

In 1955, Kon, McGillivray and Thompson (70, p. 244-267)
studying the metabolism of carotene and vitamin A in rats,

rabbits and calves showed that when 2 mg. of vitamin A
acetate suspended in Tween 40 (polyoxyethylene sorbitan
monopalmitate) were injected into rabbits, only a small
proportion of the dose was found in the blood or other
organs. When ten times the dose of vitamin A acetate was
injected within 0.5-2 minutes only one-sixth of the in-
jected dose could be recovered in the blood. After 5-7
minutes only from one-twentieth to one-thirtieth of the
dose remained there. The analysis of the carcass and
other organs showed that the injected vitamin A must have
been destroyed. The injection of Tween alone had no such
effect on the vitamin A normally present in the blood.

It was also observed that although the acetate form
of the vitamin was used for the injection, the vitamin was
recovered in all tissues apart from the liver mainly as
the alcohol form. The de-esterification of the acetate
was extremely rapid. Within 30 seconds after starting to
inject vitamin A acetate a sample of blood taken from
another part of the rabbit showed that approximately half
of the injected ester had been already converted to the
alcohol. Similar experiments with rats and calves also
indicated the rapid disappearance of vitamin A from the
blood when vitamin A in Tween was intravenously injected
into animals.

This work was further studied in rats by Pollard and

Bieri (110, p. 359-366). They checked the recovery of
vitamin A suspended in Tween when added to a homogenate
of a whole rat, severely deficient in vitamin A. Ninety-
five per cent of the added vitamin A was recovered. Each
of two vitamin A deficient rats was injected intravenously
with vitamin A acetate in Tween. The animal was immediately
killed and homogenized in less than 5 minutes. Only 64%
and 44% respectively of the injected vitamin A were recov-
ered in the homogenate of the entire carcass.

In the whole animal most of the loss occurred in the
first few minutes. After 2 hours about 44% of the dose
was left. Only traces of the vitamin appeared in the lungs,
kidneys, or adrenal glands. The vitamin A concentration
in the blood serum fell quite rapidly until a normal con-
centration was reached about 8 hours after injection. Five
minutes after injection the vitamin A level in the liver
appeared very large. There was then a rapid decrease to
a constant level during the next 2-4 hour period. This
was followed by a doubling of the vitamin A concentration
in the liver after 8 hours.

Preliminary experiments with vitamin A depleted rats
indicated that vitamin A was relatively stable when incu-
bated with a homogenate of liver, kidney, lung or spleen.
However, when vitamin A acetate was incubated with

hemolyzed whole blood, 46% of the vitamin was destroyed
at the end of 2 hours. Washed hemolyzed red cells
destroyed practically all of the vitamin A whereas the
serum was inactive.

The destructive ability was highest in the blood of
rats less than 40 days old and diminished rapidly in older
rats. This suggested that the reticulocytes which are
known to be present in large amounts in the blood of
younger animals might be responsible for the destruction
of the vitamin.

Similar studies made with other species revealed that
the blood from 14-day old-rabbits possessed a great des-
tructive activity. This largely disappeared by the time
they were 35 days old. Blood from young mice and guinea-
pigs was less active and chick blood was inactive.
Studies with rats indicated that both vitamin A acetate
and the alcohol were destroyed in approximately the same
rate. The palmitate ester and the natural esters of
vitamin A from fish oil were considerably more resistant
than the acetate or vitamin A alcohol when incubated with
rat's blood.

It was reported by Nakano (104, p. 1249-1265) that
when a homogenate of rat liver was incubated with vitamin
A the vitamin content was destroyed proportional to the

to the length of time of incubation. Vitamin A alcohol
was the most readily destroyed form followed by acetate
and least by the palmitate. Red blood cells had a similar
destructive action which was unaffected by conversion of
oxyhemoglobin to carboxyhemoglobin.

EXPERIMENTAL INVESTIGATIONS

Methods of Vitamin A Determination in Blood

Vitamin A and carotene including the presence of
small amounts of other carotenoids which do not show vita-
min A activity are found in the blood. Many of the methods
which are used to determine vitamin A in the blood are
based on chemical reactions or by the absorption band in
the ultraviolet region.

The Carr-Price method (Antimony trichloride method)

In the routine determination of vitamin A and caro-
tene in blood the most widely used method is that of
Kimble (68, p. 1055-1065) which determines the maximum
absorption at 620 mu of the blue color formed on the addi-
tion of a chloroform solution of antimony trichloride to
the vitamin in the same solvent (Carr-Price reaction 27,
p. 497-501). In this procedure the separation of carotene
and vitamin A has not been made. Carotene is determined
from the intensity of the yellow color produced after the
extraction. The total blue color obtained from the reac-
tion of vitamin A and antimony trichloride is corrected
for that portion due to the carotene. While this method
is not extremely accurate it was found very satisfactory

for blood containing less than 350 ug. of carotene per 100 ml. of blood plasma. Its comparative values may be a reliable guide to the state of nutrition.

A disadvantage of the usual Carr-Price method is due to the extreme sensitivity to minute amounts of moisture which result in turbidity from the antimony trichloride reagent. Moreover, this reagent is quite corrosive and the blue color produced is not stable. Its color fades rapidly and hence the time of measurement should not be more than 15 seconds after the antimony trichloride is added. Large samples are necessary for this method in order to obtain satisfactory results. It presents diffi-culties when attempts are made to adapt to small-scale work. However, a semi-micro adaptation of the Carr-Price method has been developed for small samples by measuring the color produced in an appropriate optical instrument.

It was reported by Brüggemann, Krauss and Tiews (23, p. 241-250) and later by Murata and Nagashima (101, p. 158-162) that the blue color formation in the Carr-Price reaction was not due to antimony trichloride but to antimony pentachloride which is usually present in the commercial sample of antimony trichloride. In order to obtain the suitable color density an excess amount of antimony trichloride is required. The latter workers found that there was a linear relationship between the

concentration of vitamin A and the intensity of absorption
produced by antimony pentachloride at 620 mu. The concen-
tration of 468 mg. of antimony pentachloride per ml. was
found to be satisfactory. The advantage of antimony
pentachloride over the trichloride was the smaller amount
of reagent required which is about 1/3150 of that of the
trichloride. An additional convenience is the fact that
the glass apparatus is not stained due to the formation
of antimony oxychloride.

Glycerol dichlorohydrin colorimetric method

This method described by Sobel and Werbin (121, p.
681-691) has used glycerol dichlorohydrin as a color re-
agent. Upon the addition of this reagent to vitamin A in
chloroform an immediate blue color appears which rapidly
changes into a more stable violet color. This method also
possesses the advantage over the Carr-Price method due to
the fact that the stable color reagent is not corrosive
and is not affected by traces of moisture. The violet
color produced is relatively stable which permits its
measurement from two to ten minutes after the reagents
are mixed. However, the violet color is less intense than
that of the antimony trichloride reaction and different
batches of the color reagent vary in sensitivity with the
method of activation (2, p. 1291-1295). This method is

also unsuitable for the micro determination.

Spectrophotometric method

This method is based on the measurement of the spec-
trophotometric absorption maximum of the vitamin at or near
328 mu (146, p. 422-425). It has been used chiefly with
materials high in vitamin A content, particularly fish-
liver oils. With substances low in vitamin A the method
fails due to the absorption at this wave length of other
substances which are usually present. To correct this
error the three point correction (25, p. 48-60) is usually
applied using an appropriate mathematical calculation.

A micro-spectrophotometric method applicable on a
micro scale has been developed by Bessey et al. (14, p.
177-188) for determining the vitamin A and carotene in
blood. This method is based on the difference in absorp-
tion at 328 mu of the nonsaponifiable fraction of the
serum before and after exposure to ultraviolet irradiation.
The irradiation presumably destroys vitamin A without ap-
preciably changing the absorption of the other substances
at 328 mu. This method can be performed with samples as
small as 60 c.mm. with satisfactory results as compared
with the Carr-Price method. However, several precautions
have to be observed. It was not applicable when high
serum carotenes were present in the sample because of the

partial destruction by ultraviolet irradiation of the
isomerized carotene (16, p. 273-279).

Separation of vitamin A from carotene

In the methods discussed so far it has not been
necessary to separate carotene from vitamin A. These
procedures have proven to be applicable for routine blood
analysis in which most of the vitamin A is predominately
in the alcohol form with moderate amounts of carotene
present. In certain experiments where the carotene con-
tent in the blood is present in large quantities the pre-
liminary saponification will remove or destroy some of the
carotenoids or non-carotenoid substances which absorb
light in the 450 mu region. Such substances would be re-
tained by the methods that do not employ a saponification
(107, p. 230-233). Nevertheless, in such a case the high
concentration of carotene will interfere with the measure-
ment of vitamin A especially with the antimony trichloride
reagent.

For more accurate experiments the carotene should be
removed from the vitamin A. Boyer, Phillips, and Smith
(22, p. 445-452) devised a method based on the differen-
tial solubilities of carotene and vitamin A in 50-60%
ethyl alcohol. The carotene is precipitated from the
alcohol solution by dilution and vitamin A remains in

solution. In this method with a high carotene content
some of the vitamin A ester will also precipitate.

Thompson, Ganguly and Kon (131, p. 50-78) provided a
simple and convenient chromatographic method for the
separation of vitamin A alcohol from carotene and the vita-
min A ester and subsequently the separation of carotene
from the ester after saponification. In this method the
authors used alumina as the absorbent and the carotene
along with the vitamin A ester was eluted from the column
by low concentrations of acetone in n-hexane whereas the
vitamin A alcohol was retained in the column and was
eluted subsequently by a stronger concentration of acetone
in n-hexane. The carotene-vitamin A ester fraction after
saponification and extraction was treated as before and
the carotene was separated from the vitamin A ester.

Another problem in the vitamin A determination results
when a low potency cis-isomer of vitamin A is incorporated
with the all-trans isomer resulting in the over-estimation
of the biological potency using the antimony trichloride
method (102, p. 453). The U.S.P. spectrophotometric
method (109, p. 941-942) gives a good estimation of the
biological potency of vitamin A.

The analytical method of choice for this experiment

Various methods for the estimation of vitamin A in calf plasma cited above were compared by Avampato and Eaton (7, p. 783-793). They concluded that within the limits of source of sample and of the amount of carotenoids present in the blood, the Carr-Price method without either saponification or separation of carotenoids is more preferable. This makes for the simplicity of method and increases its adaptability to routine work with a relatively small standard error.

Consequently the method which was used in this thesis was that of Kimble (68, p. 1055-1065) with modifications according to equipment and instruments available. This method has been used routinely to determine the vitamin A and carotene in bovine blood with satisfactory results in the Department of Agricultural Chemistry, Oregon State University, Corvallis, Oregon.

The Collection and Treatment
of Blood Samples from Calves

Twenty calves from the Oregon Agricultural Experiment Station herd were used in this study. Fourteen of the calves were Holstein, five Hereford, and one of the Angus breed and ranged in age from four days to eight months.

About 80 ml. of blood were drawn from the animal's jugular
vein with a 16 gauge hypodermic needle. The blood was
collected in a bottle containing 1.25 ml. saturated sodium
citrate per 100 ml. blood sample as the anticoagulant. A
description of the animal used in this study may be found
in Table 1.

Preparation of hemolyzed whole blood

The citrated blood sample was shaken vigorously and
transferred to a polyethylene bottle after which it was
frozen in a deep freeze refrigerator. The frozen blood
was permitted to thaw at room temperature and then re-
frozen. This process was repeated from four to five times
until the blood was completely hemolyzed.

Preparation of hemolyzed red blood cells and blood plasma

The citrated blood sample was centrifuged at 2,000
rpm for forty-five minutes after which the blood plasma
was withdrawn. The red blood cells were washed four to
five times with 0.9% saline solution and subjected to
hemolysis by repeated freezing and thawing.

47

Table 1
Bovine blood used in study

Blood No.	Animal No.	Breed	Sex	Date of Birth	Age when blood was taken	Approx. age
10	-	Hereford	Male	-	4 m. -	4 m.
11	J. 13	Hereford	Male	4-18-60	3 m. 29 d.	4 m.
12	J. 8	Hereford	Female	3-27-60	4 m. 27 d.	5 m.
13	J. 22	Hereford	Female	3-26-60	4 m. 28 d.	5 m.
14	J. 5	Hereford	Female	3-21-60	5 m. 23 d.	6 m.
15	J. 69	Angus	Female	3-17-60	5 m. 27 d.	6 m.
18	745	Holstein	Female	9-23-60	2 m. 22 d.	3 m.
19	745	Holstein	Female	9-23-60	2 m. 29 d.	3 m.
20	749	Holstein	Female	10-5-60	2 m. 17 d.	3 m.
21	Dam 669	Holstein	Female	1-9-61	4 d.	4 d.
22	Dam TX 1	Holstein	Male	1-6-61	7 d.	7 d.
23	H. 6B 5	Holstein	Male	11-18-60	1 m. 25 d.	2 m.
24	678 B_2	Holstein	Male	11-5-60	2 m. 8 d.	2 m.
25	699 B_1	Holstein	Male	9-16-60	3 m. 27 d.	4 m.
26	653 B_2	Holstein	Male	8-16-60	4 m. 27 d.	5 m.
27	739	Holstein	Female	7-25-60	5 m. 18 d.	6 m.
28	738	Holstein	Female	5-17-60	7 m. 26 d.	8 m.
29	677 B_2	Holstein	Male	10-11-60	3 m. 16 d.	4 m.
30	699 B_1	Holstein	Male	9-16-60	4 m. 11 d.	4 m.
31	653 B_2	Holstein	Male	8-16-60	5 m. 11 d.	5 m.

Preparation of non-hemolyzed whole blood and non-hemolyzed red blood cells

The carefully mixed citrated blood without vigorous shaking was referred to as the non-hemolyzed blood and the red blood cells after washing and diluting to the original volume with saline solution were referred to as the non-hemolyzed red blood cells.

Chemical Method of Vitamin A Determination

Preparation of reagents

Antimony trichloride reagent

Chloroform thoroughly washed with distilled water was dried over anhydrous potassium carbonate and distilled at 30-35°C in an all glass still under reduced pressure. The first 25 ml. of the distillate were discarded. Four ounces of antimony trichloride (Allied Chemical, New York) from a freshly opened bottle were quickly added to 500 ml. of chloroform. The mixture was frequently shaken until the antimony trichloride was all dissolved. If the solution was not clear, it was filtered. This process was carried out in a minimum of light. The concentration of this reagent was approximately 225 g. per liter. Five ml. of acetic anhydride were added to the clear solution and the reagent was stored in the dark.

Vitamin A dispersion

Crystalline vitamin A alcohol obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio was accurately weighed and dissolved in warm 95% ethanol and Tween 40 (polyoxyethylenesorbitan monopalmitate, Atlas Powder Company, Wilmington, Delaware) was added. The solution was then diluted with 0.9% saline to give a final concentration of 12.5% ethanol and 5% Tween (v/v). The concentration of the vitamin A dispersion used in the entire study was about 200 ug. of vitamin A alcohol per ml.

Preparation of standard curve of vitamin A

About 20 mg. of crystalline vitamin A alcohol were accurately weighed and dissolved in purified chloroform in a 50 ml. volumetric flask and then diluted to volume. Series of such diluted solutions in chloroform were prepared to cover the range of concentration of vitamin A up to 16 ug. of vitamin A per ml. One ml. of each solution was added to the cuvette and placed in the Coleman Universal Spectrophotometer Model 14. Nine ml. of the antimony trichloride reagent were added. The optical densities were then read exactly ten seconds after the addition of the reagent at a wave length of 620 mu. A standard curve of the concentration versus optical density

was then plotted. The correction for carotene was made by determining the absorption at 620 mu of various levels of carotene in chloroform with the antimony trichloride reagent and the curve was plotted as before. The net optical density of vitamin A was found by subtracting the optical density derived from the carotene from the total absorption.

Preparation of standard curve of beta-carotene

Crystalline beta-carotene was obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio. It was first purified by dissolving about 100 mg. in two ml. of chloroform and precipitating the beta-carotene out with 25 ml. of 90% methanol. The precipitate was filtered and washed with methanol and dried in a vacuum desiccator. Since beta-carotene is light sensitive the whole process should be carried out as much as possible in the dark. A given weight of recrystallized beta-carotene was dissolved in two ml. of purified chloroform. Redistilled Skellysolve F (b.p. 45°C) was added and diluted to 200 ml. in volumetric flask. Series of diluted solutions in Skellysolve F were prepared to cover the concentration ranging up to 2 ug. of beta-carotene per ml. The intensity of the yellow color at 450 mu was measured and the standard curve of concentration versus optical density was plotted.

Preparation of antioxidant solution

1. Propyl gallate solution. Propyl gallate (Eastman Kodak) was dissolved in a sufficient amount of water to give the final concentration of about 8 mg. of the reagent per ml.

2. Butylated hydroxytoluene (BHT) solution. A small amount of BHT (Eastman Kodak) was dissolved in warm 95% ethyl alcohol. Tween 40 was then added and the solution was further diluted with 0.9% saline solution to give a final concentration of about 5% Tween and 10-15% ethyl alcohol. The concentration of BHT was about 12 mg. per ml.

3. Alpha tocopherol solution. A small amount of alpha tocopherol (Eastman Kodak) was dissolved in the same manner as BHT. The final concentration of alpha tocopherol was about 0.3 mg. per ml.

Procedure

In most experiments 3 ml. of the blood sample, 1 ml. of the vitamin A dispersion and 6 ml. of 0.9% saline solution were placed in a 50 ml. glass stoppered centrifuge tube. To this was added 10 ml. of alcoholic potassium hydroxide solution (10 ml. of 50% aqueous potassium hydroxide solution in a 100 ml. ethyl alcohol). The

mixture was saponified at 70°C for 40 minutes. This
period of time was suggested by Bieri and Pollard (18, p.
32-44) who found that the usual 20 minute period was in-
sufficient to release the carotene from its complex with
the Tween. After cooling 10 ml. of water was added and
the mixture was extracted with Skellysolve F (b.p. 45°C)
by shaking for 15 minutes. This was followed by a mild
centrifuging for about five minutes. Since the two phases
did not separate clearly the mixture was kept in a cool
dark place or overnight in a refrigerator. Approximately
9 ml. of the solvent were removed and the carotene was
determined by reading the optical density at 450 mu in the
Coleman Universal Spectrophotometer Model 14.

An appropriate amount of the solution in the cuvette
was evaporated under vacuum at 70°C. This required less
than one minute. The residue was dissolved in 1 ml. puri-
fied chloroform. Using a syringe 9 ml. of the antimony
trichloride reagent were added quickly to the cuvette.
The optical density was then read exactly ten seconds
after the addition of the reagent at a wave length of 620
mu. A correction for the interference of carotene was
made. The amount of vitamin A present was determined from
the standard curve.

For the water-blank determination 3 ml. of distilled
water replaced the blood sample and the procedure followed

was the same in all respects. The difference between the
vitamin found by the water-blank and by the sample treated
was assumed to be the disappearance of vitamin A produced
by the effect of the blood. The percentage of vitamin A
destruction was then calculated.

$$\% \text{ destruction} = \frac{\text{Vitamin A disappearance x 100}}{\text{Vitamin A recovery from the water-blank determination}}$$

RESULTS

Experimental Proof of Method

For the blank determination water was used instead
of blood to test the effect of reagents, treatments and
the method of determination. The accurately prepared vita-
min A dispersion contained about 200 ug. of vitamin A alco-
hol per ml. Generally 1 ml. of this solution was used and
the blank determination was performed exactly as that with
the blood sample. The mean percentage of the vitamin A
recovery was 100.08 ± 0.61% as shown in Table 2. The
result is given as means with the standard error (119, p.
53-54). It is obvious that this procedure as used was
reliable. The per cent destruction of vitamin A by the
blood sample was calculated from the result obtained using
the water-blank treatment.

Since saturated sodium citrate was widely used as an
anticoagulant for blood the effect of this reagent on vita-
min A stability was tested by using the saturated reagent
instead of the blood sample. When the vitamin A recovery
was compared with that of the water blank it was found
that the saturated sodium citrate did not effect the
vitamin A stability.

A comparison was also made between the

Table 2
Recovery of vitamin A from the water-blanks

Preparation No.	Vitamin A added	Vitamin A found	Vitamin A recovery
	ug.	ug.	%
1	248.0	244.8	98.71
	248.0	244.8	98.71
	248.0	235.2	94.84
2	238.2	232.8	97.73
	238.2	237.6	99.75
	238.2	240.0	100.76
	238.2	236.4	99.24
	238.2	235.2	98.74
	238.2	240.0	100.76
	238.2	240.0	100.76
3	193.1	183.6	95.08
	193.1	189.6	98.19
4	210.9	225.6	106.97
	210.9	216.6	102.70
	210.9	218.4	103.56
	210.9	216.0	102.42
	210.9	205.2	97.30
	210.9	207.6	98.44

Table 2, continued

Preparation No.	Vitamin A added	Vitamin A found	Vitamin A recovery
	ug.	ug.	%
5	238.0	238.8	100.34
	238.0	244.8	102.86
	238.0	242.4	101.85
	238.0	248.4	104.37
	238.0	232.8	97.82
		Mean Recovery	100.08 ± 0.61[*]

[*] Standard error.

spectrophotometric method and the antimony trichloride method. The former method involved evaporating the Skellysolve F fraction at 70°C with reduced pressure to dryness and dissolving the residue in isopropyl alcohol. Three ml. of diluted isopropyl solution were put into the cuvette and the absorption curve was recorded using a Cary Recording Spectrophotometer Model 11 for the wave lengths 274-350 mu. The amount of vitamin A was calculated (41, p. 75-76) at the chosen wave lengths of 310, 325 and 334 mu.

The comparison between the two methods for the vitamin A concentration is shown in Table 3. The difference between these two methods is not significant (85, p. 132) for T = 0.454 with 8 d.f. at the 5% significance level. This means that the antimony trichloride procedure which was selected for the entire study could be applied as well as the more popular spectrophotometric method. The reason for choosing the former rather than the latter method was derived from the simplicity of method and the availability of equipment at hand. Avampato and Eaton (7, p. 791) suggested that the antimony trichloride procedure provided a high degree of precision as evidenced by their relatively small standard error. In addition it was convenient to use.

Table 3

Comparison between the antimony trichloride method
and the spectrophotometric method

	Vitamin A found	
Experiment	Antimony trichloride Method	Spectrophotometric Method
	ug.	ug.
1	3.82	3.306
2	3.37	2.907
3	4.25	3.731
4	4.25	3.771
5	7.65	7.171
Mean	4.668	4.177

By the statistical method:

The t-value is 0.454 with 8 degrees of freedom at
5% significance level. This value is outside the
critical regions so no significance difference
occurs between two methods.

Stability of Vitamin A in Bovine Hemolyzed Whole Blood

Seventeen bovine blood samples were analyzed for
their destructive effect after the addition of about 200
ug of vitamin A to the hemolyzed whole blood. It was
found that only a fraction of the whole amount of vitamin
A could be recovered. The amount which was lost during
the treatment is shown in Table 4. The mean value of the
destruction was found to be $40.9 \pm 1.2\%$ (119, p. 53-54)
as compared with the water-blank. This figure revealed
that the vitamin A destruction occurred in bovine blood
was similar to other species of animals which had already
been investigated (110, p. 364).

Effect of Duration of Time of Incubation

Different periods of incubation were introduced into
the procedure in order to verify the influence of the time
of incubation upon vitamin A stability. Three animals be-
tween four and six months of age were studied. The blood
sample after adding with the vitamin A dispersion and
0.9% saline was incubated in a constant temperature water
bath at 37°C for the various required length of time.
The reaction was stopped by the addition of alcoholic
potassium hydroxide to saponify the mixture. The times of
incubation involved were 0, 1 hour, 2 hours and 4 hours.

Table 4

Destruction of vitamin A <u>in vitro</u> by blood* from normal
calves

Blood sample No.	Vitamin A destroyed %
10	32.4
11	31.9
12	40.0
13	40.0
14	45.6
15	42.4
18	35.2
19	43.9
20	39.8
21	45.6
22	41.9
23	42.8
24	41.4
25	48.6
26	47.2
27	39.5
28	37.8
Mean value	40.9 ± 1.2**

* 3.0 ml. hemolyzed whole blood, 6.0 ml. 0.9% saline and
  1 ml. vitamin A dispersion containing about 200 ug.
  vitamin A per ml.; no incubation.
** Standard error.

The results are shown in Table 5.

The average values from these animals for the various times of incubation are 39.97, 34.73, 39.63, and 41.97% destruction respectively. At the 1 hour period the value was slightly lower than that determined at the other intervals. At any rate there was more or less the same degree of destruction. Consequently it would appear that there was no significant difference due to the different times of incubation. An analysis of variance (85, p. 163) reveals that $F = 0.5755$ at 3 and 8 d.f. at 5% significance level.

Effect of Individual Components of Blood on the Stability of Vitamin A

In order to find out which component of the blood was responsible for the destruction of vitamin A, blood samples from two animals five months of age were studied. Vitamin A dispersion was incubated for two hours with hemolyzed whole blood, with washed hemolyzed red blood cells or with blood plasma. The average values of the disappearance of vitamin A are shown in Table 6. It was found that the destruction produced by the red blood cells and by the blood plasma was 42.6 and 6.43% respectively. This shows a remarkable difference of the destructive activity between these two blood components. It appears obvious that the

Table 5

The influence of time of incubation at 37°C on the rate
of destruction of vitamin A by hemolyzed whole blood

Blood sample No.	% Vitamin A destroyed			
	No incubation 0 hour	1 hour incubation	2 hours incubation	4 hours incubation
11	31.9	28.9	30.9	32.9
14	45.6	38.6	43.7	46.8
15	42.4	36.7	44.3	46.2
Mean	39.97	34.73	39.63	41.97

Analysis of variance

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F
Treatment	84.9558	3	28.3186	0.5755*
Error	393.6467	8	49.2058	
Total	478.6025	11		

* Not significant at 5% level (85, p. 163).

Table 6

Destruction of vitamin A _in vitro_
by hemolyzed components of blood*

Experiment	System	% vitamin A destroyed		
		blood sample No. 12	blood sample No. 13	average
6	Hemolyzed whole blood	43.0	43.0	43.0
7	Hemolyzed washed red blood cells**	41.4	43.8	42.6
8	Blood plasma***	7.45	5.4	6.43

* 3.0 ml. hemolyzed sample, 6 ml. 0.9% saline and 1 ml. vitamin A dispersion containing about 200 ug. vitamin A per ml.; incubated 2 hours at 37°C.

** Red blood cells, washed 4 times with 0.9% saline, hemolyzed and 3.0 ml. sample used.

*** 3.0 ml. blood plasma from red blood cells in Experiment 7.

destruction occurring in the blood plasma is so small that
this component should not be considered as the site where
destruction takes place. The extremely high value of
vitamin A disappearance occurring in conjunction with the
red blood cells indicates that this component is the major
site of the destructive action.

Stability of Vitamin A in a Non-Hemolyzed System

The next question of interest to study was whether
the non-hemolyzed system will produce the same response as
that of the hemolyzed system. When the vitamin A disper-
sion was added without incubation to the non-hemolyzed
whole blood it was found that one fraction of vitamin A
was destroyed as that in the hemolyzed system. These
results are to be found in Table 7. Blood samples from
three calves were then used to compare the destruction of
hemolyzed and non-hemolyzed systems. The average value
for each system is shown in Table 8 with 39.6% destruction
as compared with 36.0% by the non-hemolyzed system. This
small difference cannot be considered too significant when
one accounts for the marked differences occurring in the
treatment of the two systems.

Similarly the stability of vitamin A in the non-
hemolyzed red blood cells was also tested. As shown in

Table 7

Destruction of vitamin A *in vitro*
by non-hemolyzed components of blood*

Experiment	System	% vitamin A destroyed		
		blood sample No. 19	blood sample No. 20	average
9	Non-hemolyzed whole blood	39.1	36.8	38.0
10	Non-hemolyzed washed red blood cells***	46.4**	43.8	45.1
11	Blood plasma****	0.55	1.37	1.0

* 3.0 ml. non-hemolyzed sample, 6 ml. 0.9% saline and 1 ml. vitamin A dispersion containing about 200 ug. vitamin A per ml.; no incubation.

** incubated 2 hours at 37°C.

*** Red blood cells, washed 4 times with 0.9% saline, diluted with saline to original volume; 3.0 ml. sample used.

**** 3.0 ml. blood plasma from red blood cells in Experiment 10.

Table 8

The comparison of the vitamin A stability
between the hemolyzed and non-hemolyzed blood*

Blood sample No.		% vitamin A destroyed	
		hemolyzed whole blood	non-hemolyzed whole blood
18		35.2	32.0
19		43.9	39.1
20		39.8	36.8
	Average	39.6	36.0

* 3.0 ml. blood sample, 6 ml. 0.9% saline and 1 ml.
vitamin A dispersion containing about 200 ug. vitamin A
per ml.; no incubation.

Table 7 it was found that 45.1% of the added vitamin A was destroyed by the non-hemolyzed red blood cells compared with 1% by the blood plasma. The extreme difference between the two components reveal that destructive activity in the non-hemolyzed system was also associated with the red blood cells and the blood plasma had relatively no effect on this activity.

Influence of Age on the Destructive Ability

Eight Holstein calves of different ages were used in this experiment. Their age varied from four days to eight months. The vitamin A recovery was obtained after the addition of a vitamin A dispersion to the blood sample by the usual method. As shown in Table 9 the destruction of vitamin A ranged from 37.8 to 48.6% with the mean value 43.1 ± 1.3%. Although the minimum destruction occurred for the oldest animal it would be difficult to conclude that the maximum destruction occurred at any particular age. It would appear that the correlation between the age of calves and the percentage of destruction probably does not exist in this species of animal.

Table 9

Relationship between age of calves and the stability
of vitamin A in hemolyzed blood*

Blood sample No.	Age of animals	% vitamin A destroyed
21	4 days	45.6
22	7 days	41.9
23	2 months	42.8
24	2 months	41.4
25	4 months	48.6
26	5 months	47.2
27	6 months	39.5
28	8 months	37.8
	Mean value	43.1 ± 1.3

* 3.0 ml. hemolyzed blood, 6 ml. 0.9% saline and 1 ml.
vitamin A dispersion containing about 200 ug. vitamin A
per ml.; no incubation.

Effect of Varying Amounts of Blood and Vitamin A

It should be noteworthy to determine whether increased
or decreased quantities of blood will effect the stability
of vitamin A. In order to determine this 1, 3 and 10 ml.
of hemolyzed whole blood from three different calves were
added to about 200 ug. vitamin A in Tween. The average
values of these samples from each level is shown in Table
10. The vitamin A destroyed was 40.6, 41.3, and 56.1%
for the 1, 3 and 10 ml. blood samples respectively. It is
evident that 10 ml. blood sample caused a greater percent-
age destruction than did the other two levels. The per
cent destruction at 1 and 3 ml. level was for all prac-
tical purposes the same.

Varying amounts of vitamin A were then added to a
constant level of the blood sample. To 3 ml. of hemolyzed
whole blood from three different calves was added 0.3,
0.5 and 1 ml. of vitamin A dispersion containing about
200 ug. vitamin A per ml. Table 11 shows the average re-
sults of the vitamin A destruction when different amounts
of vitamin were added. For 0.3, 0.5 and 1 ml. of the
vitamin A dispersion the destruction occurring was 40.6,
39.4, and 41.3% respectively. Thus the per cent of
destruction of vitamin A was the same when the concentra-
tion of the vitamin varied from 60 to 200 ug.

Table 10

Destruction of vitamin A _in vitro_
with varying amounts of blood*

Blood sample No.	% Vitamin A destroyed by		
	1 ml. sample	3 ml. sample	10 ml. sample
29	42.8	46.3	57.4
30	34.2	39.5	54.5
31	44.8	38.1	56.4
Average	40.6	41.3	56.1

* Indicated blood sample added to appropriate amounts of
0.9% saline, 1 ml. vitamin A dispersion containing about
200 ug. vitamin A per ml.; no incubation.

Table 11

Destruction of vitamin A in vitro
with varying amounts of vitamin A dispersion*

Blood sample No.	% Vitamin A destroyed by		
	0.3 ml. vitamin A	0.5 ml. vitamin A	1 ml. vitamin A
29	40.7	42.8	46.3
30	46.3	42.8	39.5
31	34.7	32.5	38.1
Average	40.6	39.4	41.3

* 3.0 ml. hemolyzed blood, 6 ml. 0.9% saline and indicated
quantities of vitamin A dispersion containing about
200 ug. vitamin A per ml.; no incubation.

Effect of Enzyme and Antioxidant Action
on the Stability of Vitamin A

Attempts were then made to investigate some of the
factors in the hemolyzed whole blood, which were involved
in the destruction of vitamin A. The effect of saponifica-
tion was first studied. To do this hemolyzed whole blood
and saline solution were first saponified by alcoholic po-
tassium hydroxide. When the mixture was cooled the vitamin
A dispersion was added and then mixed together. The vita-
min A left was determined in the Skellysolve F fraction.
The process of saponification before the addition of vita-
min A was then compared with the usual method. In Table 12
one observes that when vitamin A was added to the saponi-
fied hemolyzed whole blood the average destruction was 4.8%
compared with 40.2% when the addition of the vitamin
occurred in the normal manner. It would appear evident
that some enzyme system in the red blood cells was
associated with the destructivity of this vitamin.

Since vitamin A is known to be destroyed by oxidative
processes some well-known antioxidants were also used to
test this property against the vitamin A destruction.
Those tested included propyl gallate, alpha tocopherol
and butylated hydroxytoluene. An aqueous solution con-
taining 8 mg. of propyl gallate per ml. was prepared. To

Table 12

Destruction of vitamin A _in vitro_
by hemolyzed blood* as influenced by various antioxidants

Experi-ment	% Vitamin A destroyed				
	without anti-oxidant (normal)	saponi-fied**	propyl gallate	alpha tocopherol	BHT
12	39.2	0.0	6.5	-	-
13	37.7	8.3	15.4	30.4	20.5
14	45.5	4.9	15.4	34.2	24.0
15	38.2	5.9	15.4	30.4	28.1
Average	40.2	4.8	13.2	31.7	24.2

* 3.0 ml. hemolyzed blood, 6 ml. 0.9% saline, 0.5 ml.
antioxidant and 1 ml. vitamin A dispersion containing
about 200 ug. vitamin A per ml.; no incubation.

** Saponified before adding the vitamin A dispersion.

the hemolyzed whole blood was added 0.5 ml. of the propyl gallate solution. The mixture was shaken for one minute. This was followed by the addition of the vitamin A dispersion. The vitamin A recovery was determined as usual from the Skellysolve F fraction after saponification with alcoholic potassium hydroxide. The average result for four animals so treated is shown in Table 12. The average destruction of 13.2% was somewhat higher than the addition of vitamin A following saponification but was much lower than the usual method.

Butylated hydroxy-toluene (BHT) and the alpha tocopherol were also studied for their antioxidant effect. In each case 0.5 ml. of the antioxidant dispersion was used and the procedure was carried on similarly as with the propyl gallate. A water-blank was determined in each case in parallel with the sample. The destruction of vitamin A was calculated based on the recovery of vitamin A in the water-blank treatment. The average vitamin A destruction following treatment with BHT was 24.2% and with the alpha tocopherol was 31.7% as shown in Table 12.

It was found that the vitamin A recovery from the water-blank solution following the various treatments namely: the usual method, saponification, propyl gallate, BHT and alpha tocopherol was 100.5%, 102.8%, 101.8%, 86.2%

and 92.9% respectively as shown in Table 13.   From the

latter two values it would appear that BHT and alpha

tocopherol have an adverse effect on vitamin A under these

conditions.

Table 13

Percentage of the vitamin A recovery from the water-blank
after treatment with antioxidants

No.	Treatment	Vitamin A recovery from the water-blank
		%
1	Without antioxidant	100.5
2	Saponification prior to the addition of vitamin A dispersion	102.8
3	With propyl gallate	101.8
4	With BHT	86.2
5	With alpha tocopherol	92.9

DISCUSSION

The destruction of vitamin A by blood _in vivo_ has
been recorded in human subjects (84, p. 747-761), in rats,
rabbits and calves (70, p. 244-267). It has been exten-
sively studied _in vitro_ in rats by Pollard and Bieri (110,
p. 359-366) and more recently by Nakano (104, p. 1249-
1265). LePage and Pett (84, p. 747-761) after the admin-
istration of large doses of vitamin A to humans found by
spectrographic examination, a vitamin A oxidation product,
probably vitamin A epoxide, with a maximum absorption at
275 mu in the blood extracted with cyclohexane-ethanol
mixture. Contrarily in the present study no peak was found
from 274 to 310 mu in an isopropanol extracted sample
using the Cary Recording Spectrophotometer. If oxidative
products had been formed, they should show some peaks in
this region as suggested by Bolomey (20, p. 331-335). It
is rather doubtful that the oxidative products existed in
this destructive system.

Surface-active agents consisting of fatty acid esters
of the polyoxyethylene sorbitan have been widely used to
prepare the vitamin A aqueous dispersions. One of the most
commonly used is Tween 40 (polyoxyethylene sorbitan mono-
palmitate) which is easily dispersed and a good carrier
for the rapid absorption of the fat soluble vitamin after

injection into the experimental animal (133, p. 108-110; 70, p. 244-267). This solution is also very stable as shown by Bieri (15, p. 327-334) who demonstrated that in the absence of antioxidants vitamin A acetate is stable for 28 days in an aqueous solution with Tween 40 whether stored under air or nitrogen.

This effect was also noted in this present study. A stock vitamin A dispersion did not change its concentration from day to day even when it was kept for two weeks. This may be noted from the water-blank recovery shown in Table 2. It is to be added that the vitamin dispersion was stored in a cold dark place as well. It is possible that when the vitamin A dispersion is added to the hemolyzed whole blood at least some parts of the vitamin may be protected from the coming oxidative action by the stabilizing effect of Tween 40 acting as an antioxidant.

The effect of Tween upon the hemolysis was described by Sobel, Rosenberg and Engel (120, p. 184-185). They found that the red cells in 100 ml. of heparinized whole blood of rabbits resisted as much as 200 mg. of sorethytan laurate without hemolysis. The administration of the aqueous dispersion in such manner that only 30-40 mg. of the dispersing agents were present in 100 ml. of blood allowed a wide safety margin. However, even higher quantities of Tween than mentioned cause no hemolysis in rats

or rabbits but was fatal to calves (70, p. 262). The Tween content of the dose of vitamin A injected into calves had to be reduced to 20 mg. per pound body weight instead of 80 mg. per pound.

Pollard and Bieri (110, p. 359-366) prepared a vitamin A aqueous dispersion containing 5% Tween (v/v) in their studies with rats both in vivo and in vitro. This procedure was also followed in this study. They reported that non-hemolyzed whole blood had slight activity, possibly some hemolysis occurred when the Tween solution was added. In this investigation it was found that the non-hemolyzed whole bovine blood had the same destroying effect on the vitamin as did the hemolyzed system. It may be possible that the same concentration of Tween which caused the slight or no hemolysis in the rat's blood caused considerable hemolysis in the bovine blood as described by Kon, McGillivray and Thompson (70, p. 255-262). Table 8 shows the tendency for the non-hemolyzed and the hemolyzed systems to have the same destructive action.

It has been demonstrated that in vivo there occurred the destruction of vitamin A in Tween by blood when the vitamin was injected intravenously into calves (70, p. 258). At first sampling at five minutes after injection the blood vitamin A alcohol and vitamin A ester increased rapidly. The vitamin A ester then decreased markedly and

fell to its initial value within one hour. The vitamin A also decreased between 5 and 15 minutes and then increased, but only about half as much as five minutes after the injection. It remained nearly constant for five hours and then decreased to its initial value. The total vitamin A present in the liver, lungs, and kidneys was only one-tenth of the dose injected. A considerable loss of vitamin A from the blood can be shown in vitro by this investigation (Table 4) which indicates that about 40.9 ± 1.2% of vitamin A is destroyed. This value is nearly the same as that recorded for rat's blood and about half as much as that for rabbit's blood (110, p. 364).

The finding as shown in Table 6 and Table 7 shows that red blood cells either in the hemolyzed or non-hemolyzed system are responsible for the destruction of vitamin A. Since the destructive effect shown is extremely small in the plasma it should be concluded that an insignificant amount of this activity resides in the plasma. This finding is in accordance with the work of Pollard and Bieri (110, p. 361-362) and of Nakano (104, p. 1249-1265) who found similar results with rats. This finding is not unreasonable as it is known that red blood cells contain many enzymes or factors which presumably may be involved with the destructivity.

It is rather difficult to state that the incubation

time had any influence on the vitamin added as is shown in
Table 5. The slight decreased activity after one hour
incubation did not change the general pattern of the
destruction. It would seem from this data that the per-
centage of the vitamin destroyed remained unchanged regard-
less of the time of incubation. This is not in agreement
with the remarkable increased destructivity of the blood
of rats with different incubation times (110, p. 362-363)
which showed that after 15-20 minutes of incubation about
50% of the vitamin A was destroyed and after one hour
about 95% was destroyed.

Pollard and Bieri (110, p. 363-364) suggested that
with rats there exists a correlation between the vitamin
A destroyed and the amount of reticulocytes present in
the blood. The evidence for this came from the fact that
blood from young rats produced a higher destructivity than
that from older rats. The former contained more reticulo-
cytes than the latter. In this study this remarkable dif-
ference was not found with Holstein calves from the age of
four days to eight months (Table 9). The somewhat higher
destructivity by the four and five months old bovine blood
did not change the general pattern of constant stability.
Since the relationship between age and vitamin A destruc-
tion was not apparent it was unnecessary to study further

the content of reticulocytes in individual animals for the
purpose of finding the relation between reticulocytes and
the destructive activity.

When the concentration of vitamin A dispersion was
kept constant as shown in Table 10, the relatively large
increases in amounts of blood sample caused a somewhat
higher vitamin A destruction. The destruction in bovine
blood, however, is much more constant than that reported
for rat's blood (110, p. 362-364) where 74.6%, 45.0% and
95.0% destruction occurred for 0.5, 1.0 and 1.5 ml. of
blood respectively. This may be related by some means to
the daily requirement of the animal. Table 11 shows that
within the range of 60-200 ug. of vitamin A, blood will
cause more or less the same percentage of destruction. In
the rabbit, it had been previously demonstrated that when
20 mg. of vitamin A acetate were injected intravenously
into the animal, only 0.67-1.0 mg. of vitamin A was re-
covered in the blood. When treated similarly with one-
tenth of this dose, a small proportion of vitamin A was
still found in the blood (70, p. 251-253). Thus it would
seem that a constant proportion of the added vitamin A
acetate was destroyed as some of the vitamin was found in
the blood regardless of the concentration injected. This
mechanism as well as the action reported in these two
tables is not yet well understood and beyond the scope of
this thesis.

The chief component of the erythrocyte is hemoglobin which is a chromoprotein composed of globin and heme. Oleic acid and polyunsaturated fatty acids are the predominant components of the total fatty acids present in whole blood. The dienoic acid was the highest in the polyunsaturated fatty acid group (108, p. 427-429). It is already well known that the red blood cell is the main site for various enzyme systems. Enzyme activity of the erythrocyte and reticulocytes have also been studied (117, p. 222-235). Hemoglobin has been reported to play a role as the catalytic agent for the oxidation of unsaturated fatty acids (129, p. 721-733) as well as vitamin A and carotene (128, p. 475-478).

A mechanism in the hemolysis of the erythrocyte is believed to be the penetration of the lysin into the cell wall and the breakdown of the membrane lipoprotein cholesterol complex (114, p. 450-464). The hemolyzate of the erythrocyte showed a catalytic effect on the oxidation of the unsaturated fatty acids, probably due to the presence of hemoglobin (28, p. 773-779).

In this present study, when saponification occurs before the addition of vitamin A dispersion, the alcoholic potassium hydroxide may inhibit enzyme activity as well as denature blood protein and hence only a slight destruction follows such a treatment. The average value from four

animals treated was 4.8% destruction (Table 12). This
constitutes quite definite proof of the stability of vita-
min A with reference to the enzyme system or catalytic
effect of hemoglobin in the erythrocyte. Since there are
many kinds of trace elements and different levels of con-
centration in the red blood cell (59, p. 846-854), it
might also be expected that the catalytic effect of trace
elements also might be involved in the destruction of
vitamin A.

It has been reported by many workers that alpha toc-
opherol as well as some phenolic compounds propyl gallate,
butylated hydroxytoluene (BHT), butylated hydroxyanisole
have powerful antioxidant properties by inhibiting the
unsaturated fatty acid oxidation catalyzed by hemoglobin
(28, p. 773-779; 135, p. 957-964). Collier and McRae (28,
p. 773-779) have suggested that the high concentration
of hemoglobin together with oxygen might cause the oxi-
dation of the unsaturated fatty acid in the cell membrane.
Hence there is the possibility that alpha tocopherol may
play a role in protecting components of the erythrocytic
membrane from oxidation catalyzed by hemoglobin. This
suggestion seems reasonable since the concentration of
oleic acid and dienoic acids in blood plasma are higher
than that in the red blood cells and even the saturated

fatty acids and the tetraenoic acids in the former are less than in the latter (108, p. 427-429).

In this study the antioxidant effect of some of these well-known phenolic compounds as propyl gallate, BHT, and alpha tocopherol have been tested. An advantage of the propyl gallate over the others was its water solubility and ease of preparation. The other two had to be prepared in an aqueous dispersion similar to that of vitamin A. No attempt was made to determine a critical limit for each of the added antioxidants prepared. The main purpose was to demonstrate qualitatively the antioxidant effect of these antioxidants on preventing the destruction of vitamin A by hemolyzed whole blood. The effect of alpha tocopherol has been reported by Tappel (128, p. 475-478) studying the inhibition of unsaturated fatty acid oxidations catalyzed by hematin compounds. It can be concluded that the function of alpha tocopherol may reduce the secondary vitamin A cooxidation by inhibiting the primary oxidation of oleic acid.

With the procedure used in this study it was thought that the first addition of the antioxidant would inhibit some of the mechanism of the enzymatic effect or the catalytic effect of the hemoglobin in the hemolyzed whole blood. As is shown in Table 12, among the three

antioxidants tested, propyl gallate showed a remarkable
effect in decreasing the destructivity as compared with
the usual method. The other two antioxidants, although
they showed a somewhat decreasing effect, were much less
effective than propyl gallate. In fact the total inhibit-
ing effect of alpha tocopherol differed only slightly to
that without the antioxidant present. Furthermore, BHT
and alpha tocopherol showed some influence on the vitamin
A stability under these conditions since the vitamin A
recovery from the water-blank with the addition of BHT and
alpha tocopherol was 86.2% and 92.9% respectively as shown
in Table 13.

Negative results for the antioxidants have been
reported by Rapoport, Gerischer-Mothes and Nieradt (112,
p. 174-187) who were unable to show the inhibitory effect
of tocopherol for the unsaturated fatty acid oxidation in
the blood hemolyzate but tocopherol remarkably accelerated
the reaction. It might be possible that the tocopherol
used in this study was not in the proper concentration.
It has been observed that the tocopherol function is most
effective at lower levels of concentration with decreased
efficiency at higher levels (126, p. 176-180; 91, p. 162-
165; 51, p. 105-107). Dam (30, p. 195-196) has indicated
that a certain concentration of antioxidant may give its

optimum effect while an increase above this concentration may lead to a pro-oxidant effect.

SUMMARY

1. Blood samples from 20 cows of various ages were used to study the stability of vitamin A in hemolyzed blood.

2. When two hundred micrograms of vitamin A in Tween were added to the blood hemolyzate, the average vitamin A destruction was found to be $40.9 \pm 1.2\%$.

3. The vitamin A destruction by blood was constant regardless of time of incubation up to 4 hours.

4. Both the hemolyzed and non-hemolyzed whole blood caused about the same extent of destruction of vitamin A.

5. The red blood cells either hemolyzed or non-hemolyzed were found to be the site which was associated with the destructive activity.

6. No significant difference in the percentage of vitamin A destruction was found in blood from calves whose age ranged from four days to eight months.

7. If the blood hemolyzate was first saponified no significant destruction of the added vitamin A was found. It is evident that some factors involving an enzyme system or catalytic effect of hemoglobin may be responsible for vitamin A stability.

8. Among the antioxidants tested, propyl gallate proved to be more effective than butylated hydroxytoluene

and alpha tocopherol in stabilizing vitamin A under
conditions described.

BIBLIOGRAPHY

1. Alexander, J. and T. W. Goodwin. A demonstration of
        the conversion of carotene into vitamin A.
        British Journal of Nutrition 4:421-423. 1950.

2. Allen, R. S. and S. W. Fox. Reactions of activated
        glycerol dichlorohydrin with vitamin A.
        Analytical Chemistry 22:1291-1295. 1950.

3. Ames, S. R., W. J. Swanson and P. L. Harris. Bio-
        chemical studies on vitamin A. 15. Biopotencies
        of geometric isomers of vitamin A aldehyde in
        the rat. Journal of the American Chemical
        Society 77:4134-4136. 1955.

4. Ames, S. R., W. J. Swanson, H. A. Risley and P. L.
        Harris. Vitamin A aldehydes-metabolism, bio-
        potency and toxicity. Federation Proceedings
        13:174. 1954.

5. Arens, J. F. and D. A. Van Dorp. Synthesis of some
        compounds possessing vitamin A activity. Nature
        157:190-191. 1946.

6. Arens, J. F. and D. A. Van Dorp. Activity of vitamin
        A acid in the rat. Nature 158:622-623. 1946.

7. Avampato, J. E. and H. D. Eaton. Comparison of meth-
        ods for the estimation of vitamin A in calf
        plasma. Journal of Dairy Science 36:783-793.
        1953.

8. Bäck, K. Effect of some metal ions on the stability
        of natural vitamin A. Acta Chemica Scandinavica
        10:334-335. 1956.

9. Bäck, K. Effect of some pro-oxidants and antioxidants
        on stability of natural vitamin A. Acta Chemica
        Scandinavica 13:60-74. 1959.

10. Ball, S., F. D. Collins, P. Dalvi and R. A. Morton.
        Studies in vitamin A. 11. Reactions of retinene
        with amino compounds. Biochemical Journal 45:
        304-307. 1948.

11. Ball, S., J. Glover, T. W. Goodwin and R. A. Morton. Conversion of retinene to vitamin A *in vivo*. Biochemical Journal 41:xxiv. 1947.

12. Ball, S., T. W. Goodwin and R. A. Morton. Studies on vitamin A. 5. The preparation of retinene-vitamin A aldehyde. Biochemical Journal 42: 516-523. 1948.

13. Baumann, C. A., B. M. Riisong and H. Steenbock. Fat-soluble vitamins. XLII. The absorption and storage of vitamin A in the rat. Journal of Biological Chemistry 107:705-715. 1934.

14. Bessey, O. A., O. H. Lowry, M. J. Brock and J. A. Lopez. The determination of vitamin A and carotene in small quantities of blood serum. Journal of Biological Chemistry 166:177-188. 1946.

15. Bieri, J. G. Stability of aqueous and oily vitamin A acetate and carotene solutions. Journal of Nutrition 44:327-334. 1951.

16. Bieri, J. G. and M. O. Schultze. Comments on the microspectrophotometric determination of vitamin A and carotene in blood. Archives of Biochemistry and Biophysics 34:273-279. 1951.

17. Bieri, J. G. and C. J. Pollard. Studies of the *in vitro* conversion of carotene to vitamin A. Texas Reports on Biology and Medicine 11:402-411. 1953.

18. Bieri, J. G. and C. J. Pollard. Studies of the site of conversion of beta carotene injected intravenously into rats. British Journal of Nutrition 8:32-44. 1954.

19. Bolomey, R. A. Oxidative decomposition of vitamin A. I. Stability of vitamin A toward oxidation and irradiation. Journal of Biological Chemistry 169:323-329. 1947.

20. Bolomey, R. A. Oxidative decomposition of vitamin A. II. Absorption spectrophotometry of oxidized vitamin A. Journal of Biological Chemistry 169: 331-335. 1947.

21. Bone, J. F. A technic for aspiration liver biopsy
        in dairy cattle. North American Veterinarian
        35:747-752. 1954.

22. Boyer, P. D., P. H. Phillips and J. K. Smith. The
        separation of carotene from vitamin A for the
        determination of vitamin A in blood plasma.
        Journal of Biological Chemistry 152:445-452.
        1944.

23. Brüggemann, J., W. Krauss and J. Tiews. Zur Methodik
        der chemischen Vitamin A-und D-Bestimmung mit
        elektrophilen Metallhalogeniden. I. Allgemeines
        und Methodik mit Antimonpentachlorid.
        Zeitschrift für Analytische Chemie 146:241-250.
        1952.

24. Buxton, L. O. Tocopherols as antioxidants for vita-
        min A in fish liver oil. Industrial and Engin-
        eering Chemistry 39:225-232. 1947.

25. Cama, H. R., F. D. Collins and R. A. Morton. Studies
        in vitamin A. 17. Spectroscopic properties of
        all-trans vitamin A and vitamin A acetate
        analysis of liver oils. Biochemical Journal 50:
        48-60. 1951-1952.

26. Capper, N. S., I. M. W. McKibbin and J. H. Prentice.
        Carotene and vitamin A. The conversion of
        carotene into vitamin A by fowl. Biochemical
        Journal 25:265-274. 1931.

27. Carr, F. H. and E. A. Price. Colour reactions
        attributed to vitamin A. Biochemical Journal
        20:497-501. 1926.

28. Collier, H. B. and S. C. McRae. Antioxidants as in-
        hibitors of linoleate oxidation catalyzed by
        plant lipoxidase and by hemolysates of human
        erythrocytes. Canadian Journal of Biochemistry
        and Physiology 33:773-779. 1955.

29. Crook, E. M. and M. K. El-Marsafy. Association of
        carotene with serum proteins. Biochemical
        Journal 57:viii. 1954.

30. Dam, H. Vitamin E as an _in vivo_ antioxidant. Inter-
     national Union of Nutritional Sciences. Meeting
     5:195-213. 1952. (Experimentia. Supplementum
     1.)

31. Davies, A. W. and A. N. Worden. The stability of
     vitamin A in animal feeding stuffs. Journal of
     the Science of Food and Agriculture 5:107-112.
     1954.

32. Davis, R. E. and L. L. Madsen. Carotene and vitamin
     A in cattle blood plasma with observations on
     reproductive performance at restricted levels of
     carotene intake. Journal of Nutrition 21:135-
     146. 1941.

33. Diven, R. H., O. F. Pahnish, C. B. Roubicek, E. S.
     Erwin and H. M. Page. Vitamin A and carotenoid
     inter-relationships in bovine plasma and liver.
     Journal of Dairy Science 43:1632-1637. 1960.

34. Dowling, J. E. and G. Wald. Vitamin A acid and the
     function of vitamin A. Proceedings of the
     National Academy of Sciences 46:587-608. 1960.

35. Drummond, J. C., H. J. Channon and K. H. Coward.
     Studies on the chemical nature of vitamin A.
     Biochemical Journal 19:1047-1067. 1925.

36. Duliere, W., R. A. Morton and J. C. Drummond. The
     alleged relation of carotin to vitamin A.
     Journal of the Society of Chemical Industry
     48:316T-321T. 1929.

37. Dzialoszynski, L. M., E. M. Mystowski and C. P.
     Stewart. The mode of occurrence of carotene and
     vitamin A in human blood plasma. Biochemical
     Journal 39:63-69. 1945.

38. Eden, E. and K. C. Seller. The absorption of vitamin
     A in ruminants and rats. Biochemical Journal
     44:264-267. 1949.

39. Eden, E. and K. C. Seller. Hydrolysis and esterifi-
     cation of vitamin A during absorption. Bio-
     chemical Journal 46:261-266. 1950.

40. Elliot, R. F. Studies on the site of absorption and conversion of carotene to vitamin A in the dairy calf. Journal of Dairy Science 32:711-712. 1949.

41. Embree, N. D., S. R. Ames, R. W. Lehman and P. L. Harris. Determination of vitamin A. In: Methods of biochemical analysis. Vol. 4. New York, Interscience Publishers Inc., 1957. p. 43-98.

42. Ensminger, M. E., M. W. Galan and W. L. Slocum. Problems and practices of American cattleman. Pullman, 1955. 89 p. (Washington. Agricultural Experiment Station. Bulletin no. 562)

43. Erwin, E. S., T. R. Varnell and H. M. Page. Relationship of vitamin A and carotene to bovine serum. Proceedings of the Society for Experimental Biology and Medicine 100:373-375. 1959.

44. Evers, N. The permanence of vitamin A in cod-liver oil as shown by the color test. Quarterly Journal of Pharmacy and Pharmacology 2:556-565. 1929.

45. Fazakerley, S. and J. Glover. The provitamin A activity of some possible intermediates in beta carotene metabolism. Biochemical Journal 65: 38P-39P. 1957.

46. Fishwick, M. J. and J. Glover. The metabolism of uniformly C^{14} labelled beta carotene in the rat. Biochemical Journal 66:36P-37P. 1957.

47. Ganguly, J. and H. J. Deuel. Intracellular distribution of vitamin A esterase activity in rat liver. Nature 172:120. 1953.

48. Garbers, C. F., J. Gillman and M. Peisach. The transport of vitamin A in rat serum with special reference to the occurrence of unidentified metabolites of vitamin A in the rat. Biochemical Journal 75:124-132. 1960.

49. Glover, J., T. W. Goodwin and R. A. Morton. Studies in vitamin A. 6. Conversion in vivo of vitamin A aldehyde (retinene$_1$) to vitamin A$_1$. Biochemical Journal 43:109-114. 1948.

50. Glover, J., and E. R. Redfearn. The mechanism of the transformation of beta carotene into vitamin A in vivo. Biochemical Journal 58:xv-xvi. 1954.

51. Golumbic, C. The autoxidative behavior of vegetable and animal fats. Oil and Soap 20:105-107. 1943.

52. Gray, E. L. and J. D. Cawley. The state of vitamin A in the liver of the rat. II. The effect of feeding the vitamin over extended periods. Journal of Nutrition 23:301-307. 1942.

53. Gray, E. L., K. Morgareidge and J. D. Cawley. Intestinal absorption of vitamin A in the normal rat. Journal of Nutrition 20:67-74. 1940.

54. Groot, E. H. Oxidative phenomena in dilute solutions of substances with a system of conjugated double bonds. 1. Vitamin A acetate. Recueil des Travaux Chimiques des Pays-Bas 68:185-190. 1949. (Abstracted in Nutrition Abstracts and Reviews 19:44-45. 1949-1950)

55. Groot, E. H. and J. P. Colpa. Oxidative phenomena in dilute solutions of substances with a system of conjugated double bonds. 3. Further studies on vitamin A acetate. Recueil des Travaux Chimiques des Pays-Bas 68:871-878. 1949. (Abstracted in Nutrition Abstracts and Reviews 19:839. 1949-1950)

56. Halpern, G. R. Spectrophotometric changes during oxidation of vitamin A oils. Industrial and Engineering Chemistry. Analytical Edition 18:621-625. 1946.

57. Halverson, A. W. and E. B. Hart. Factors affecting the stability of the vitamin A from cod liver oil in cereal feeds. Journal of nutrition 40:415-427. 1950.

58. Halverson, A. W. and C. H. Hendrick. Effects of
        minerals and other dietary ingredients upon
        vitamin A stability in stored poultry diets.
        Poultry Science 34:355-359. 1955.

59. Herring, W. B., B. S. Leavell, M. Paixao and J. H.
        Yoe. Trace metals in human plasma and red blood
        cell. American Journal of Clinical Nutrition
        8:846-854. 1960.

60. Hickman, K. C. D., M. W. Kaley and P. L. Harris.
        Covitamin studies. 1. The sparing action of
        natural tocopherol concentrates on vitamin A.
        Journal of Biological Chemistry 152:303-311.
        1944.

61. High, E. G. Further antioxidant studies concerned
        with the metabolism of carotene and vitamin A.
        Archives of Biochemistry and Biophysics 60:456-
        462. 1956.

62. High, E. G., H. B. Bright and J. R. Powell. In vitro
        hydrolysis and esterification of vitamin A.
        Federation Proceedings 15:556. 1956.

63. High, E. G., J. R. Powell and H. B. Bright. In vivo
        and in vitro hydrolysis of vitamin A esters and
        esterification of vitamin A alcohol. Federation
        Proceedings 16:388. 1957.

64. High, E. G., H. C. Smith, Jr., H. H. Taylor and S. S.
        Wilson. Antioxidant studies concerned with the
        metabolism of carotene and vitamin A. Journal
        of Biological Chemistry 210:681-686. 1954.

65. Hubbard, R. and G. Wald. Cis-trans isomers of vita-
        min A and retinene in the rhodopsin system.
        Journal of General Physiology 36:269-315. 1952-
        1953.

66. Hume, E. M. and I. Smedley-Maclean. Some further
        notes on the relation of carotene to vitamin A.
        The Lancet 218:290-292. 1930.

67. Hunter, R. F. and E. G. E. Hawkins. Vitamin A
        aldehyde. Nature 153:194. 1944.

68. Kimble, M. S. The photo-colorimetric determination
        of vitamin A and carotene in human plasma.
        Journal of Laboratory and Clinical Medicine
        8:1055-1065. 1939.

69. Kon, S. K. and S. Y. Thompson. Site of conversion
        of carotene to vitamin A. British Journal of
        Nutrition 5:114-119. 1951.

70. Kon, S. K., W. A. McGillivray and S. Y. Thompson.
        Metabolism of carotene and vitamin A given by
        mouth or vein in oily solution or aqueous dis-
        persion to calves, rabbits and rats. British
        Journal of Nutrition 9:244-267. 1955.

71. Krause, R. F. and C. Alberghini. The hydrolysis of
        vitamin A ester. Archives of Biochemistry 25:
        396-400. 1950.

72. Krause, R. F. and L. T. Powell. The in vivo hydroly-
        sis and esterification of vitamin A. Archives
        of Biochemistry and Biophysics 44:57-62. 1953.

73. Krause, R. F. and P. L. Sanders. Metabolism of C^{14}
        labeled beta carotene in the rat. Proceedings
        of the Society for Experimental Biology and
        Medicine 95:549-551. 1957.

74. Krinsky, N. I. The enzymatic esterification of
        vitamin A. Journal of Biological Chemistry 232:
        881-894. 1958.

75. Krinsky, N. I., D. G. Cornwell and J. L. Oncley.
        Vitamin A, carotenoids and plasma lipoproteins.
        Federation Proceedings 15:113. 1956.

76. Krinsky, N. I., D. G. Cornwell and J. L. Oncley.
        The transport of vitamin A and carotenoids in
        human plasma. Archives of Biochemistry and
        Biophysics 73:233-246. 1958.

77. Krishnamurthy, S., P. Seshadri Sastry and J. Ganguly.
        Studies on vitamin A esterase. II. The hydroly-
        sis of natural vitamin A ester with the living
        animal. Indian Journal of Medical Research 45:
        391-394. 1957. (Abstracted in Biological
        Abstracts 32:no.33197. 1958.)

78. Kuhn, R. and K. Livada. Über den Einfluss von
    Seitenketten auf die Oxydationsvorgänge im
    Tierkörper. Modellversuche zum biologishen
    Abbau der Carotinfarbstoffe. Hoppe-Seyler's
    Zeitschrift für Physiologische Chemie 220:235-
    246. 1933.

79. Laughland, D. H. The hydrolysis of vitamin A esters
    by rat liver homogenates. Archives of Bio-
    chemistry and Biophysics 73:95-99. 1958.

80. Laughland, D. H. and W. E. J. Phillips. The effect
    of sodium bentonite administration on vitamin A
    metabolism in the rat. Canadian Journal of
    Biochemistry and Physiology 32:593-599. 1954.

81. Laughland, D. H. and W. E. J. Phillips. The adsorp-
    tion of vitamin A by sodium bentonite. Canadian
    Journal of Biochemistry and Physiology 32:610-
    620. 1954.

82. Lemley, J. M., R. A. Brown, O. D. Bird and A. D.
    Emmett. Absorption and storage of vitamin A
    in the liver of the rat. Journal of Nutrition
    33:53-64. 1947.

83. Lemley, J. M., R. A. Brown, O. D. Bird and A. D.
    Emmett. The effect of mixed tocopherols on the
    utilization of vitamin A in the rat. Journal of
    Nutrition 34:205-218. 1947.

84. LePage, G. A. and L. B. Pett. Absorption experiments
    with vitamin A. Journal of Biological Chemistry
    141:747-761. 1941.

85. Li, Jerome C. R. Introduction to statistical infer-
    ence. Ann Arbor, Michigan, Edwards Brothers,
    Inc., 1957. 553 p.

86. Mahadevan, S., S. Krishnamurthy and J. Ganguly. The
    mode of absorption of vitamin A across the in-
    testine of rats. Archives of Biochemistry and
    Biophysics 83:371-375. 1959.

87. Mattson, F. H., J. W. Mehl and H. J. Deuel, Jr.
    Studies on carotenoid metabolism. VII. The site
    of conversion of carotene to vitamin A in the
    rat. Archives of Biochemistry 15:65-73. 1947.

88. McGillivray, W. A. The conversion of carotene to
        vitamin A in sheep and cattle. Australian
        Journal of Scientific Research. Series B.
        Biological Sciences 4:370-376. 1956.

89. McGillivray, W. A., S. Y. Thompson and N. A. Worker.
        Further studies on the metabolism by rats of
        intravenously administered aqueous dispersions
        of carotenoid pigments. British Journal of
        Nutrition 10:126-134. 1956.

90. McGugan, W. A. and D. H. Laughland. The vitamin A
        esterase activity of rat liver homogenate.
        Archives of Biochemistry and Biophysics 35:
        428-434. 1952.

91. Miles, S. M. C., E. M. Erickson and H. A. Mattill.
        Tocopherol vs. tocopherol acetate as a "sparer"
        of vitamin A. Proceedings of the Society for
        Experimental Biology and Medicine 70:162-165.
        1949.

92. Miller, M. W., V. Joukovsky and N. Hokenstad. The
        effect of manganese sulfate on the stability of
        vitamin A and D of cod liver oil when stored in
        mixed feeds. Poultry Science 21:200-202. 1942.

93. Moore, L. A., M. H. Berry and J. F. Sykes. Carotene
        requirements for the maintenance of a normal
        spinal fluid pressure in dairy calves. Journal
        of Nutrition 26:649-658. 1943.

94. Moore, L. A. and J. F. Sykes. Cerebrospinal fluid
        pressure and vitamin A deficiency. American
        Journal of Physiology 130:684-689. 1940.

95. Moore, L. A., J. F. Sykes, W. C. Jacobson and H. G.
        Wiseman. Carotene requirements for Guernsey and
        Jersey calves as determined by spinal fluid
        pressure. Journal of Dairy Science 31:533-538.
        1948.

96. Moore, T. The relation of carotin to vitamin A. The
        Lancet 217:380-381. 1929.

97. Moore, T. Vitamin A and carotene. VI. The conversion
        of carotene to vitamin A _in vivo_. Biochemical
        Journal 24:696-702. 1930.

98. Moore, T. The effect of vitamin E deficiency on the
    vitamin A reserves of the rat. Biochemical
    Journal 34:1321-1328. 1940.

99. Morton, R. A. Chemical aspects of the visual process.
    Nature 153:69-71. 1944.

100. Morton, R. A. and T. W. Goodwin. Preparation of
     retinene in vitro. Nature 153:405-406. 1944.

101. Murata, T. and J. Nagashima. Colorimetric estima-
     tion of vitamin A and beta carotene with
     antimony pentachloride. The Journal of
     Vitaminology 6:158-162. 1960.

102. Murray, T. K. and J. A. Campbell. Estimation of the
     biological potency of vitamin A. Federation
     Proceedings 20:453. 1961.

103. Murthy, S. K., S. Mahadevan, P. Seshadri Sastry and
     J. Ganguly. Fatty acid specificity for the
     esterification of vitamin A and cholesterol by
     intestinal and pancreatic enzymes in rats.
     Nature 189:482. 1961.

104. Nakano, M. An experimental study on destruction of
     vitamin A. Journal of Osaka City Medical Center
     9:1249-1265. 1960. (Abstracted in Nutrition
     Abstracts and Reviews 31:60. 1961.)

105. Olcott, H. S. and D. C. McCann. Carotenase. The
     transformation of carotene to vitamin A in
     vitro. Journal of Biological Chemistry 94:
     185-193. 1931.

106. Osborne, T. B. and L. B. Mendel. The vitamins in
     green foods. Journal of Biological Chemistry
     37:187-200. 1919.

107. Parrish, D. B., G. H. Wise and J. S. Hughes. Vita-
     min A and carotenoid in the blood serum of
     dairy cattle. Chemical methods for determina-
     tion. Analytical Chemistry 20:230-233. 1948.

108. Patil, V. S. and N. G. Magar. Fatty acids of human
     blood. Biochemical Journal 74:427-429. 1960.

109. Pharmacopeia of the United States of America. 15th
          revision, U.S.P. XV. Easton, Pennsylvania,
          Mack Publishing Co., 1955. 1178 p.

110. Pollard, C. J. and J. G. Bieri. The destruction of
          vitamin A by blood. British Journal of Nutri-
          tion 12:359-366. 1958.

111. Pollard, C. J. and J. G. Bieri. Esterification of
          vitamin A by an acetone powder from pancreas.
          Archives of Biochemistry and Biophysics 87:9-
          12. 1960.

112. Rapoport, S., W. Gerischer-Mothes and C. Nieradt.
          Häminkatalysierte Oxydation ungesättigter
          Fettsäuren im Blut anämischer Kaninchen.
          Hoppe-Seyler's Zeitschrift für Physiologische
          Chemie 300:174-187. 1955.

113. Reid, B. L., H. K. Daugherty and J. R. Couch. The
          stability of vitamin A in mixed feeds and pre-
          mixes. Poultry Science 34:603-608. 1955.

114. Rideal, E. and F. H. Taylor. On hemolysis and
          hemolytic acceleration. Proceedings of the
          Royal Society of London. Series B. Biological
          Sciences 148:450-464. 1958.

115. Ronning, M. and C. B. Knodt. The absorption of
          vitamin A natural esters and of carotene by
          young male Holstein calves. Journal of Dairy
          Science 35:283-291. 1952.

116. Rosenberg, A. and A. E. Sobel. In vitro conversion
          of carotene to vitamin A in the isolated small
          intestine of the rat. Archives of Biochemistry
          and Biophysics 44:320-325. 1953.

117. Rubinstein, D., P. Ottolenghi and O. F. Denstedt.
          The metabolism of the erythrocyte. XIII. Enzyme
          activity in the reticulocyte. Canadian Journal
          of Biochemistry and Physiology 34:222-235.
          1956.

118. Smith, E. L., F. A. Robinson, B. E. Stern and F. E. Young. Studies in the stability of vitamin A and D. III. The effect of light on vitamin A. Biochemical Journal 33:207-212. 1939.

119. Snedecor, G. W. Statistical Methods. 3d ed. Ames, Iowa, The Iowa State College Press, 1940. 422 p.

120. Sobel, A. E., A. Rosenberg and E. Engel. Intravenous injection of fat-soluble vitamins and transfer to milk of injected vitamin A. Journal of Nutrition 48:183-192. 1952.

121. Sobel, A. E. and H. Werbin. Spectrophotometric study of a new colorimetric reaction of vitamin A. Journal of Biological Chemistry 159:681-691. 1945.

122. Stallcup, O. T. and H. A. Herman. In vitro studies on the conversion of carotene to vitamin A in dairy calves. Journal of Dairy Science 33:237-242. 1950.

123. Steenbock, H. and P. W. Boutwell. Fat-soluble vitamine. III. The comparative nutritive value of white and yellow maizes. Journal of Biological Chemistry 41:81-96. 1920.

124. Steenbock, H., M. T. Sell, E. M. Nelson and M. V. Buell. The fat-soluble vitamine. Journal of Biological Chemistry 46:xxxii. 1921.

125. Swick, R. W. and C. A. Baumann. Effect of certain tocopherols and other antioxidants on the utilization of beta carotene for vitamin A storage. Archives of Biochemistry and Biophysics 36:120-126. 1952.

126. Swift, C. E., W. G. Rose and G. S. Jamieson. Factors affecting the stability of cottonseed oil. A study of the antioxygenic activity of alpha tocopherol. Oil and Soap 19:176-180. 1942.

127. Tappel, A. L. The inhibition of hematin-catalyzed oxidation by alpha tocopherol. Archives of Biochemistry and Biophysics 47:223-225. 1953.

128. Tappel, A. L. Studies of the mechanism of vitamin E action. II. Inhibition of unsaturated fatty acid oxidation catalyzed by hematin compound. Archives of Biochemistry and Biophysics 50: 473-485. 1954.

129. Tappel, A. L. Unsaturated lipide oxidation catalyzed by hematin compound. Journal of Biological Chemistry 217:721-733. 1955.

130. Thomas, J. W., W. C. Jacobson and L. A. Moore. Factors unrelated to vitamin A intake that influence plasma vitamin A concentration. Journal of Dairy Science 35:679-686. 1952.

131. Thompson, S. Y., J. Ganguly and S. K. Kon. The conversion of beta carotene to vitamin A in the intestine. British Journal of Nutrition 3:50-78. 1949.

132. Thompson, J. N. and G. A. J. Pitt. Vitamin A acid and hypervitaminosis A. Nature 188:672-673. 1960.

133. Tomarelli, R. M., J. Charney and F. W. Bernhart. Utilization of intramuscularly injected carotene. Proceedings of the Society for Experimental Biology and Medicine 63:108-110. 1946.

134. Troitskii, G. V. Products of auto-oxidation of vitamin A. Biokhimiia 15:485-489. 1950. (Abstracted in Nutrition Abstracts and Reviews 21:296. 1951-1952)

135. Tsen, C. C. The protection action of tocopherol against hemolysis of rat erythrocytes by dialuric acid. Canadian Journal of Biochemistry and Physiology 38:957-964. 1960.

136. Van Dorp, D. A. and J. F. Arens. Biological activity of vitamin A acid. Nature 158:60. 1946.

137. Verandani, P. T., G. J. Wright, G. Wolf and B. C.
     Johnson. Metabolism of radioactive vitamin A
     and vitamin A acid. Federation Proceedings
     20:452. 1961.

138. Wald, G. Carotenoids and the visual cycle. Journal
     of General Physiology 19:351-371. 1935-1936.

139. Wall, M. E. and E. G. Kelly. Stability of carotene
     and vitamin A in dry mixtures. Industrial and
     Engineering Chemistry 43:1146-1150. 1951.

140. Week, E. F. and F. J. Sevigne. Vitamin A utiliza-
     tion studies. 1. The utilization of vitamin A
     alcohol, vitamin A acetate and vitamin A
     natural esters by the chicks. Journal of
     Nutrition 39:233-250. 1949.

141. Wiese, C. E., J. W. Mehl and H. J. Deuel, Jr.
     Studies on carotenoid metabolism. VIII. The
     in vitro conversion of carotene to vitamin A
     in the intestine of the rat. Archives of
     Biochemistry 15:75-79. 1947.

142. Wodsak, W. Die Verwendbarkeit der Vitamin A-Säure
     zur Vitaminierung fetthaltiger Lebensmittel.
     Fette:Seifen:Anstrichmittel 61:672-675. 1959.
     (Abstracted in Nutrition Abstracts and Reviews
     30:434. 1960.

143. Worker, N. A. The effect of complete hepatectomy
     on the utilization by rats and rabbits of in-
     travenously administered aqueous dispersions
     of carotene. British Journal of Nutrition 10:
     169-175. 1956.

144. Worker, N. A. Site of conversion of carotene into
     vitamin A in the rat. Further studies on
     aqueous dispersions administered intravenously.
     British Journal of Nutrition 11:44-47. 1957.

145. Worker, N. A. Studies on the in vitro conversion
     of beta-carotene into vitamin A in tissues
     from the rat, guinea pig and sheep. British
     Journal of Nutrition 13:400-418. 1959.

146. Zacheile, F. P. and R. L. Henry. Ultraviolet absorption of vitamin A in various solvents. Industrial and Engineering Chemistry. Analytical Edition 14:422-425. 1942.